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The Initial Fate of a Trace Amount of Intravenously Administered Selenite

By

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Abstract Selenite injected intravenously was rapidly taken up by the erythrocytes after which it was ejected into the plasma in which the selenium became bound to protein (mainly to albumin) The selenium was then taken up by other cells mainly in the liver which maximally contained radioselenium 15 min after the injection Following this a gradual discharge into the plasma occurred

Key words Selenite – distribution – erythrocytes – liver – mice

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The accumulation and distribution of selenium in certain tissues after the administration of selenite has been demonstrated many times (HEINRICH & KELSEY 1955, GANTHER 1965, JACOBSSON & HANSSON 1965, HOPKINS *et al* 1966) However the transfer mechanisms are not known although the subsequent distribution has been observed There have previously been reports of the accumulation of selenium in the erythrocytes (BUESCHER *et al* 1961, KUTTLER *et al* 1961, WRIGHT & BELL 1963) and recently it has been shown that there is a very rapid initial uptake of selenite in the erythrocytes, after which the selenium is pumped out from the cells (LEE *et al* 1969)

The present investigation has been undertaken to determine the acute fate of intravenously injected selenite and the participation of the erythrocytes in the metabolism of selenite *in vivo* in mice

Material and methods

Forty male albino mice weighing 25 g were injected intravenously into the tail vein with a trace amount of labelled sodium selenite (30 ng sodium selenite in 0 2 ml 0 9 % saline total ^{75}Se activity 50 nci) Thereafter the mice were killed after various intervals by decapitation and 0 5 ml blood was drawn directly from the heart into a heparinized syringe The organs (liver, kidneys and the alimentary canal including

the stomach and the intestines with contents and the pancreas) were dissected and put into tubes for the measurement of radioactivity

The 0 5 ml samples were immediately put into Eppendorf centrifuge tubes containing 1 ml saline +4° The tubes were centrifuged (12,000×g/min) and the supernatants sucked off and put into other tubes

The radioactivity of the organs, blood cells and the diluted plasma were measured by a NaI scintillation counter The netto impulses were compared with the amount of radioselenite injected and the percentages plotted as shown in figs 1 and 2 The *livers of four additional mice decapitated after 15 minutes were perfused with Ringer s* solution before measurement of the radioactivity

To demonstrate to which plasma proteins the selenium is attached five other mice were injected with radioselenite 0 5 μci (containing 2 μg sodium selenite / 0 2 ml saline) A heparinized plasma sample (about 0 2 ml) was drawn by heart puncture after 5 and 45 minutes (heparin 1 mg/ml) The blood samples were centrifuged and paper electrophoresis was run with 6 μl amounts of the plasma (Beckman model R system Run according to the instruction manual)

After the run some strips were stained with amidoblack to locate the proteins and others cut into 6 mm transverse strips for the measurement of the radioactivities

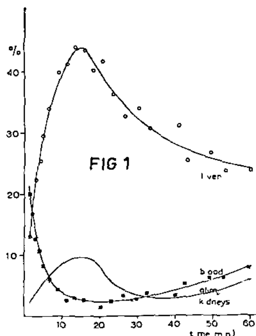


Fig 1 The distribution of intravenously injected trace amount of ^{75}Se sodium selenite in organs and blood of mice The percentages from the total addition are given for the whole liver and kidneys alimentary canal including stomach and intestines with the pancreas and a blood sample representing about 28 % of the total circulating blood

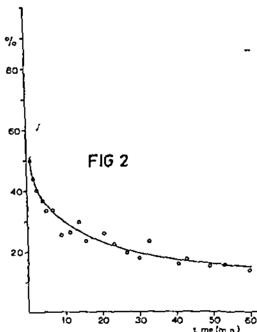


Fig 2. The distribution of intravenously injected ^{75}Se sodium selenite between the blood cells (unbroken line) and the plasma (broken line)

Results

From fig 2 it can be seen that the intravenously injected selenium (as selenite) has been taken up very rapidly by the red blood cells (at 1 min more than 50 % inside the cells) Thereafter there was a gradual expulsion process

Fig 1 shows that the selenium disappears from the blood simultaneously with the selenium ejected from the erythrocytes (cf fig 2) The selenium which is handled by the blood cells is taken up by other cells in the organism mainly by the liver the maximum radioselenium content occurring at 15 minutes This radioactivity could not significantly be reduced with perfusion of the liver There is also an expulsion mechanism in these organs and the selenium again accumulates in the plasma after which concentrations of the drug gradually become increased in the intestine kidneys and urine The blood contents in fig 1 must not be taken as such because only 0 5 ml blood was analyzed and the total blood content can be estimated to be about 1 8 ml

Electrophoresis followed by gamma counting showed that in the 5 min samples 45 % had been lost during the electrophoresis and in the 45 min

samples about 38 % was lost The radioactivities were distributed in the protein peaks The notable difference between these two series of samples was that there was more selenium bound to albumin in the 5 min samples (21 % from the total activity on the strip) than in the later 45 min samples (12 %)

Discussion

The results show that the intravenously administered selenite is handled by the erythrocytes before it enters other cells in the organism The selenium is possibly transferred by serum proteins, mainly albumin, from the ery throcytes to the other cells as concluded from the albumin attached selenium concentration difference in the 5 and 45 min samples It has been assumed that the selenium is attached between two protein sulphurs (LEE *et al* 1969, McCONNEL 1970, JENKINS & HIDIROGLOU 1970), or that the labile complex on albumin could be a glutathione selenium complex (SANDHOLM 1972, unpublished results) The radioactivity is shifted from the erythrocytes to other organs mainly liver, from which it is gradually expelled The form of the selenium at this time is not known but after this stage some selenium excretion is observed, mainly in the form of methyl selenides (McCONNEL & PORTMAN 1952, GANTHER 1966, BYARD 1969, PALMER *et al* 1969)

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The Metabolism of Selenite in Cow Blood *in Vitro*

By

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Abstract The transfer of selenite via the erythrocytes to the plasma was investigated using Se^{75}-sodium selenite and cow blood The selenite was initially fluxed into the erythrocytes by a cyanide sensitive mechanism During the uptake period (within 1 min) the erythrocytes consumed oxygen and it can be concluded that selenium is reduced and catalyzes the electron flow to the oxygen The lack of electrons thus produced is balanced by the oxygenation of GSH The release is dependent on GSH and it is possible that selenium is ejected from the erythrocytes as a glutathione complex which is fixed to the SH of plasma albumin

Key words Selenite – erythrocytes – metabolism – cow blood *in vitro*

It has been shown that the red blood cells of humans and several animal species concentrate selenium *in vivo* and *in vitro* (Smith *et al* 1937, Buescher *et al* 1961, Kuttler *et al* 1961, Jensen *et al* 1963, Wright & Bell 1963, Lee *et al* 1969) A special initial phase of reaction has been shown in human erythrocytes, the selenium is first fluxed into the cells and thereafter pumped out (Lee *et al* 1969) Selenium is bound to special plasma proteins in the living animal and the binding proteins vary with the time (McConnel *et al* 1960, Jenkins *et al* 1969)

The present investigation has been undertaken to study the transfer and binding mechanism of selenium when introduced to plasma proteins via blood cells

Experiment 1 The transfer of selenium into the erythrocytes using different selenite concentrations

Methods

Different sodium selenite solutions were prepared in 0 9 % NaCl con-

taining selenium 25, 2 5, 0 25 etc mM Cow blood was taken in heparin (heparin 1 mg/ml) and cooled to 25°

The experiments were started within 10 minutes of the blood collection by adding 9 ml of blood to test tubes containing 1 ml of the sodium selenite solution (final blood selenium concentrations 0–2 5 mM, final PCV 23 5 %) Each tube contained 0 1 μci Se^{75} sodium selenite as a tracer The tubes were shaken continuously and a 1 ml sample was taken from each tube after 1, 2, 5, 10, 15, 20, 30, 45 and 60 minutes and pipetted into a 1 8 ml Eppendorf centrifuge tube containing 0.5 ml of 0 9 % saline Centrifugation was started immediately (12,000 × g/1 min) The supernatant was sucked off and the radioactivity in the tubes measured with a NaI scintillation counter This experiment was done in triplicate

Result

The results are shown in fig 1 With small selenite concentrations (0 00025,A and 0 0025 mM B) it was found that the selenium was rapidly taken up by the blood cells (within 1–2 min) and thereafter the ejection exceeded the uptake With higher concentrations of selenite (0 025,C and 0 25 mM,D) the bulk of the selenium remained in the cells Using very high

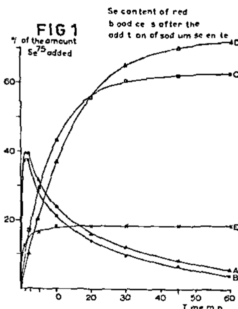


Fig 1 The erythrocyte ^{75}Se content after addition of various amounts of labelled selenite Selen te additions (mM whole blood) A 0 00025 B 0 0025 C, 0 025 D 0 25 E 2 5

selenium concentrations (2 5 mM,E) the uptake was restricted the cells contained selenium 2 0 mM

Experiment 2 The changes of acid soluble thiols during selenium uptake and expulsion

Methods

Experiment 1 was repeated and the acid soluble thiols in the erythrocytes were analyzed by a modified Ellman method (ELLMAN 1959) The proteins were precipitated with sulphosalicylic acid (final concentration 5 %, w/v) and the acid soluble thiols, mainly reduced glutathione (GSH) allowed to react with the Ellman reagent dithionitrobenzoic acid (DTNB) Half ml samples of the selenite-blood solutions were taken at similar intervals as in Experiment 1 and put into Eppendorf tubes containing 1 ml of 0 9 % NaCl The tubes were centrifuged, the supernatants sucked off and the tubes filled ad 1000 μl with ice cold 5 7 % sulphosalicylic acid (875 μl) The tubes were shaken and left at 4° for one hour and then centrifuged after which 200 μl aliquots of the supernatants were taken into 10 ml test tubes to which were added 5 ml of phosphate buffer (0 3 M, pH 6 8) and a calculated amount (about 45 μl) of 1 N-NaOH to neutralize the acid Ellman

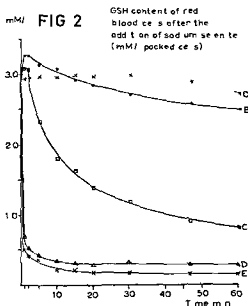


Fig 2 The GSH content of erythrocytes after addition of various amounts of Na_2SeO_3 Selenite concentrations (mM whole blood) O no selenite B 0 0025 C, 0 025 D 0 25 E 2 5

reagent, 100 µl of DTNB 1 mg/ml phosphate buffer, was added to each tube Glutathione standards containing 0 5, 1, 2, 3 and 4 mM were processed similarly to the blood cells The absorptions were measured spectrophotometrically at 412 nm using 1 cm light path

Result

The results are plotted in fig 2 The erythrocytes originally contained acid soluble thiols (reduced glutathione) 2 9 mM, curve 0 When small selenite concentrations (B,C) were used, the reduced glutathione was somewhat elevated during the initial phase of uptake after which it gradually fell When large amounts of selenite (D,E) were used the GSH fell very rapidly to a constant low level

It can be calculated from curve B that GSH fell about 0 4 mM (packed cells) from the initial level in the blood cells When the total selenium addition was 0 0025 mM whole blood and if this is inside the cells, the packed cell content is about 0 01 mM, the assessed GSH decrease was 40 times as compared with processed selenium, if it is assumed that all the selenium had been taken up and expelled

Experiment 3 The effect of inhibitors on selenite transfer

Methods

The previous experiments were repeated after the cells had been treated for 3 minutes with 10^{-3} M cyanide, azide, 2,4 dinitrophenol, iodoacetamide and 10^{-5} M azoester ($C_6H_5N = NCOOCH_3$) The last one is known to oxidate the erythrocyte GSH (Kosower *et al* 1969) To determine the manner in which the cell GSH oxidation affects the selenium influx, an additional experiment was carried out in which the azoester was added at a time when the erythrocytes contained a maximum amount of selenium (i e after 1 minute, curve B fig 1 and fig 3) The removal of reduced glutathione was checked by the Ellman method

Result

The uptake was inhibited by cyanide (8 % in the cells after 60 minutes) The release was inhibited by the SH reagents iodoacetamide and azoester The reduced glutathione fell after azoester treatment giving a curve not unlike curve E in fig 2 The selenium content in the experiments based on azoester are shown in fig 3 This shows that pretreatment of the cells with azoester (curve G) delays the initial influx and inhibits the efflux The addition of azoester after 1 minute, results in inhibition of the expulsion while the uptake continues (curve F) The addition of 2,4 dinitrophenol or azide does not markedly affect either process

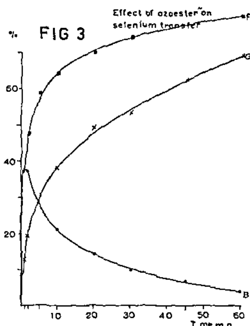


Fig 3 The effect of azoester on the accumulation of selenite by the erythrocytes (Selenite 0 0025 mM blood) F azoester added at 1 min, G, blood treated for 3 min with azoester before selenium addition, B control (no azoester)

Experiment 4 Oxygen consumption of blood during selenium transfer

Methods

Oxygen consumption of the blood after the addition of selenite was measured by constant pressure manometry The gas consumption in the apparatus was measured using a detergent water diaphragm in a horizontal micro burette The experiments were made at 25°

A 9 ml sample of heparinized cow blood was put in a 100 ml reaction vessel with similar selenite solutions as in Experiments 1 and 2 The vessel was connected to the microburette (inner diameter 1 5 mm) containing a detergent bubble The blood and the selenite were mixed and the vessel shaken vigorously during the experiment The movement of the diaphragm was noted The experiments were repeated using KOH absorption

Result

Air consumption was rapid during the initial 30 seconds Total consumption was completed in 45 seconds, after which there was a slight gas volume

increase which for the most part could be prevented by using KOH absorption From the air volume decrease during the first 45 seconds it was calculated that in sample A the oxygen consumption was 2 7 μmol O_2/10 ml blood (PCV 23 5, Hb 9 0 after the addition of selenite) Other figures B 2 9, C 3 5, D 6 0 and E 7 0 μmol (the letters refer to those in experiments 1 & 2 and figs 1 & 2)

When cyanide or azide pretreated cells were used (incubated for 10 min , 1 mM concentrations), the selenite induced oxygen consumption was initially low, but then the rate increased slowly, so that the same oxygen consumption as in experiments without these heme reagents was reached after about one hour

Experiment 5 The binding of selenium to plasma proteins

Methods

a) To 10 ml of heparinized cow blood was added radioactive sodium selenite (0 0025 μM/ml, total activity 0 1 μci PCV 29 5 after selenite addition) After 30 minutes at 25° the blood was centrifuged (7000 × g/10 min at 4°) One ml samples of the plasma were taken and put into dialyzing tubes and dialyzed overnight against phosphate buffered saline (PBS) pH 7 3 Similarly radioselenite was added directly to the same volume of plasma and dialyzed against PBS after which the radioactivity was measured from the dialyzing bags by NaI scintillation
b) To 1 ml of heparinized blood was added radioselenite 0 0025 μmol/ml, total activity 0 1 μci The blood was centrifuged after 30 minutes and 6 μl samples were taken Paper electrophoresis was started immediately and after 24 hours incubation at 25° (Model R paper electrophoresis system, Beckman Schleicher & Schull 2043a Mgl paper strips, 0 1 M veronal buffer pH 8 6, 0 4 mA/strip/16 hours) After the run some strips were stained with amidoblack to locate the protein peaks while other strips were cut transversally into 6 mm strips and the radioactivities of these measured

Results

When the selenium had passed through the erythrocytes (30 min at 25°) and dialyzed, 44 % of the added selenium was found inside the dialyzing tube after intensive dialysis for 24 hours When the same amount was put directly into plasma only 0 6 % remained inside the bag When a paper electrophoresis was made from the former dialyzed samples, 54 % more of the selenium was lost (freed from the protein during the electrophoresis) The radioactivity was distributed in the protein areas, though the main part was in the fibrinogen globulin region The resulting Se distribution was

albumin 12 0 %, α_1 10 9 %, α_2 9 1 %, β globulin fibrinogen-γ globulin 68 % Protein distribution was found to be albumin 43 %, α_1 4 8 %, α_2 5 6 % and β globulin fibrinogen-γ globulin 46 6 % If the plasma was introduced to electrophoresis immediately after removal of the erythrocytes it contained more radioselenium in albumin (22 %) than the sample introduced 24 hours later (14 %)

Experiment 6 The binding of selenite to plasma proteins in the presence of GSH

Methods

Radioselenite and tritiated GSH were added separately and simultaneously to heparinized plasma In the latter instances only one of them was labelled After electrophoresis the radioactivity from various areas was measured

To 100 µl of heparinized cow plasma was added 20 µl 0 01 M $Na_2Se^{75}O_3$ (total activity 0 1 µci) and 20 µl of physiological saline To the second plasma sample was added 20 µl of 0 01 M tritiated GSH (total activity 0 5 µci) and saline Other samples containing the same amounts of selenite and GSH added simultaneously were labelled alternately After 5 minutes the paper electrophoresis was started After a 16 hours' run 6 mm strips were cut transversally and those containing tritiated glutathione counted by liquid scintillation For the latter the pieces were placed into counting vessels containing a toluene scintillation cocktail (2 vol toluene 1 vol Triton X100 containing 0 7 % PPO (w/v) and 0 03 % POPOP (w/v))

After shaking overnight the bottles were counted with a Wallack DECEM 314 instrument The maximum counting efficiency was 35 %

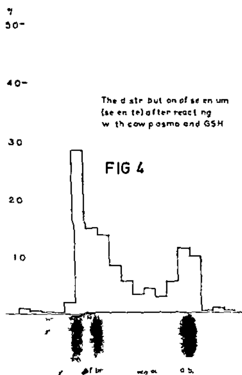
Result

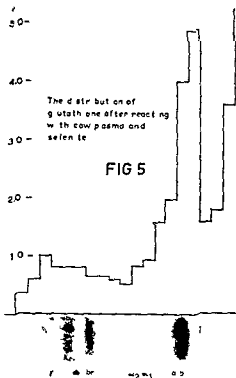
The averages of three parallel analyses are shown in figs 4–6 No binding was observed when only selenite was left with the plasma When GSH was allowed to react with the plasma 20 % of the total radioactivity was found in the protein area as shown in fig 6 (the peak to the right of the albumin is non bound glutathione)

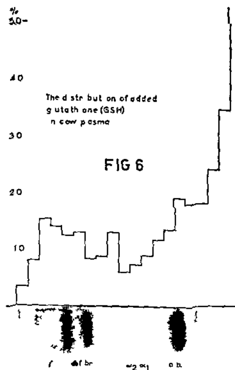
When selenite and GSH were added to the plasma together, there was a special glutathione binding to the albumin (fig 5) Using radioselenite and GSH, the selenium also became fixed to the albumin (fig 4) The selenium at the site of application was possibly Se_0 which had been produced

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Figs 4 5 6 The incorporation of selenite (fig 4) and glutathione (fig 5) into plasma proteins after reacting equimolarly in heparinized cow plasma Fig 6 demonstrates the binding of GSH when reacting alone with plasma The percentages are calculated from the total additions


50-
40-
30
20
10
The d str but on of se en um
(se en te) after react ng
w th cow p asma and GSH
FIG 4


50-
40 -
30 -
20 -
10 -
The d str but on of
g utath one after react ng
w th cow p asma and
selen te
FIG 5


%
50-
40
30
20
10
The d str but on of added
g utath one (GSH)
n cow p asma
FIG 6

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Competitive Antagonism Between Phenoxybenzamine and Acetylcholine in Isolated Rat Atria

By

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(Received October 20, 1972, Accepted December 15, 1972)

Abstract In isolated rat atria kept at 32°, phenoxybenzamine 10^{-6} M and 2 5 × 10^{-6} M added 5 min before acetylcholine (ACh), caused an almost parallel shift to the right and no fall in the maximum response in the log dose response curves for ACh In the present experiments, these results are indicative of a reversible antagonism between phenoxybenzamine and ACh

Key words Isolated rat atria – phenoxybenzamine – acetylcholine – competitive antagonism

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It has previously been shown that phenoxybenzamine, an α-adrenergic blocking agent belonging to the β-haloalkylamine class of drugs, possesses an atropine-like activity In the isolated guinea-pig atrium, the drug prevented the effects of vagus stimulation and of acetylcholine (ACh) (BENFEY & GREEFF 1961, BENFEY & GRILLO 1963) and in the isolated toad bladder, guinea-pig rectum and ileum the effect of added ACh (BOYD *et al* 1963, COOK 1971) In addition, it has been demonstrated that the changes in the isolated rat atrial electrogram induced by ACh are prevented by phenoxybenzamine, and that atrial flutter and fibrillation produced by a combination of the electrical stimulation and ACh are converted into sinus rhythm after the addition of the drug (LANDMARK & REFSUM 1973)

The question arises whether the blocking action of phenoxybenzamine in isolated rat atria is competitive and hence strictly "atropine-like", or merely due to an unspecific effect The present experiments were undertaken in order to test these possibilities

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* Student Research Fellow, The Norwegian Research Counsil for Science and Humanities

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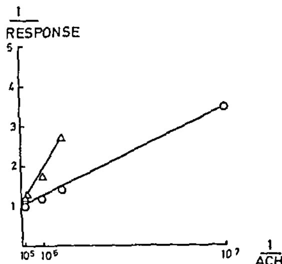


Fig 2 Double reciprocal plot of acetylcholine plottet against the reciprocals of the effects elicited by acetylcholine in the absence (O——O) and presence of phenoxybenzamine 2.5×10^{-6} M (Δ——Δ)

inotropic effect of ACh being virtually unchanged This maximal response elicited by ACh was achieved within the same time interval in the absence and presence of phenoxybenzamine When the reciprocals of ACh were plotted against the reciprocals of the effects elicited by ACh in the absence and presence of phenoxybenzamine 2.5×10^{-6} M it was found that the regression lines had a common intercept with the ordinate (fig 2) Fig 3 shows a typical example of the anticholinergic effect of phenoxybenzamine

The maximum ACh response varied from 300 to 560 mg In order to compare the results this value has been called 100 per cent in each ex

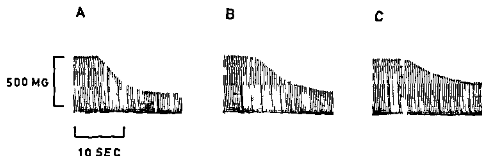


Fig 3 The negative inotropic effect of acetylcholine 5×10^{-7} M in the absence (A) and the presence of phenoxybenzamine 10^{-6} M (B) and 2.5×10^{-6} M (C)

periment The maximum reduction of contractile force after addition of ACh was 85 3 ± 2 6 (mean ± S E M) per cent of the amplitude of the contractions

Discussion

The results presented in this study show that phenoxybenzamine 10^{-6} M and 2 5 × 10^{-5} M produces a shift to the right along the ACh dose axis without significantly changing either its slope or asymptote These curves are characteristic of a reversible competitive antagonism Phenoxybenzamine is generally regarded as a blocking agent for α adrenergic receptors, but this drug and other compounds belonging to the β-haloalkylamine class of drugs, have also been shown capable of inhibiting the responses to a wide variety of other agonists These includes histamine (FURCHGOTT 1955, NICKERSON 1956, COOK 1971), 5-hydroxybenzamine (COOK 1971) and ACh (BOYD *et al* 1963, BENFEY & GRILLO 1963)

The blockade caused by phenoxybenzamine and the related compounds has generally been considered irreversible or non-competitive However, NICKERSON (1956) has reported that low doses of GD-121 (N-(2-chloroethyl) methyl-1 naphthalein methylamine HCl = OF-109, SY-14) (0 0005 µg/ml and 0 01 µg/ml) shifted the histamine dose-response curve by at least 2 log units along the agonist dose axis without changing either its slope or asymptote, and COOK (1971) found that the histamine dose-response curve underwent a parallel shift of about 0 5 log units in the presence of phenoxybenzamine 5 9 × 10^{-7} M Higher concentrations of GD-121 and of phenoxybenzamine depressed both the slope and asymptote The hypothesis proposed by NICKERSON (1956) to explain irreversible antagonism of GD-121 is that only a small fraction of the available receptors need to interact with the agonist to elicit the maximum response This has been referred to as the receptor reserve hypothesis However, the idea of a "reserve of receptor" has recently been rejected by several investigators (MORAN & TRIGGLE 1969, COOK 1971) An alternative hypothesis proposed *to explain a parallel shift of the dose response curve after irreversible antagonism* is the so called two site hypothesis (ARIENS *et al* 1960, MORAN & TRIGGLE 1969) In the case of phenoxybenzamine the blockade of this drug should result from an interaction at a site in the neighbourhood of the receptor which interferes with the receptor, but does not inactivate it thus giving rise to an antagonism which appears competitive in nature and limits the access of agonist to the receptor without rendering it non-functional This would produce a shift in the dose-response curve which appears competitive Higher concentrations of phenoxybenzamine would inactivate the receptor, thus leading to a decrease in maximum response (COOK 1971)

In interpreting the antagonistic effects of the β-haloalkylamine class of drugs, it is also necessary to consider the temperature at which the preparation is kept and the length of time of equilibration between antagonist and reacting structure at the receptor site NICKERSON (1956) and ROCHA E SILVA et al (1972) have demonstrated that when the guinea-pig ileum is exposed to GD-121 and phenoxybenzamine for as long as 10 and 15 min respectively, the inhibition against histamine becomes persistent If the preparation is kept at 37°, the intensity of the blockade remains constant even after repeated washings with warm Tyrode (37°), but if the preparations is submitted to repeated washings with Tyrode kept at 2–4°, the sensitivity progressively increases BENFEY & GRILLO (1963) have described an antagonism of phenoxybenzamine on the inhibiting action of ACh on the isolated atrium This antagonism was, in contrast to atropine, slow in onset and very slowly reversible When atropine and phenoxybenzamine were added simultaneously, the action of ACh was quickly inhibited, and the effect disappeared after withdrawal, indicating a protection of the ACh receptor against phenoxybenzamine by atropine This indicates that phenoxybenzamine and atropine react with the same receptors

In our experiments the concentrations of the antagonist were relatively low, and the contact time with the atrium only 5 min These facts may explain why a drug which has been classified as an irreversible antagonist is capable of producing a shift to the right of the ACh dose response curve without reducing its maximum response

Acknowledgements

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Contractile Responses to Phenylephrine and Carbachol in Circular Rabbit Fundus Strips at Varying External K^+ and Ca^{++} Concentrations

By

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(Received November 6, 1972, Accepted January 15, 1973)

Abstract Both phenylephrine and carbachol produced dose-dependent contraction in isolated circular strips from the fundus of rabbit stomachs The threshold concentration required to produce contraction (phenylephrine 10^{-6} M, carbachol 2×10^{-7} M) remained the same when the external K^+ concentration ($[K^+]_o$) was altered from zero to 94 4 mM, and the external Ca^{++} concentration ($[Ca^{++}]_o$) from zero to 3 75 mM The magnitude of the contractions produced by the two drugs was greatly altered however, and these alterations were best seen in the responses to the highest concentrations used (phenylephrine 10^{-4} M, carbachol 2×10^{-5} M) The responses to carbachol 2×10^{-5} M were significantly ($P < 0\,05$) greater than the response to phenylephrine 10^{-4} M at all $[K^+]_o$ and $[Ca^{++}]_o$ other than $[Ca^{++}]_o = 0$ In order to compare the influence of varying $[K^+]_o$ and $[Ca^{++}]_o$ on the responses to the two drugs the per cent alterations in the responses were studied The contractions produced by both phenylephrine and carbachol increased when $[K^+]_o$ was raised from zero to 23 8 mM ($P < 0\,005$) but were reduced when $[K^+]_o$ was raised further (to 47 2 and 94 4 mM) ($P < 0\,05$) Increasing $[Ca^{++}]_o$ gave increasing responses to both drugs The reduction in the response to phenylephrine 10^{-4} M when $[K^+]_o$ was reduced from 5 9 mM to zero was less marked than the reduction in the response to carbachol 2×10^{-5} M ($0\,1 > P > 0\,05$ for the difference), but the increase in the response to phenylephrine when $[Ca^{++}]_o$ was increased from 0 31 to 3 75 mM was significantly greater ($P < 0\,05$) than the increase in the response to carbachol It is suggested that the increase in response with increasing $[K^+]_o$ from zero to 23 8 mM may be due to a secondary increase in available Ca^{++}, while the reduction in response at higher $[K^+]_o$ may be due to the depolarization which these $[K^+]_o$ produce

Key words α adrenergic receptors – cholinergic receptors – gastric motility – gastric smooth muscle – potassium – calcium

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In a previous paper it was reported that phenylephrine – an adrenergic α receptor stimulating agent – nearly always produces a sustained contraction in circular rabbit fundus strips (HAFFNER 1972) The same effect is obtained with carbachol, and the initial phase of both these contractile

responses appears to be associated with an increase in K^+-efflux (HAFFNER *et al* 1972) This may indicate that alteration in the membrane permeability to K^+ with a subsequent alteration in the ratio between intra- and extra-cellular K^+ is one step in the excitation contraction process

The aim of the present study was to determine how variations in the external K^+ concentration ($[K^+]_o$) influence the contractile response to phenylephrine in circular rabbit fundus strips, and whether such variations had the same influence on the contractile response to carbachol

The effect of variations in the external Ca^{++} concentration ($[Ca^{++}]_o$) on the responses to phenylephrine and carbachol have been studied for comparison

Material and Methods

The stomachs of 26 male white rabbits were used for the present investigation Circular fundus strips without mucosa were prepared and mounted as described previously (HAFFNER 1972) in modified Krebs solution at 38°, bubbled with 95 % O_2 and 5 % CO_2 (pH=7 4) Two separate baths each containing two strips were used The strips in one bath (randomely chosen each time) served as controls these strips were exposed to the same concentrations of drug as the test preparations at the same time but whereas the K^+ or Ca^{++} concentration of the test bath was changed between successive dose response curves it was kept constant in the control bath

The standard Krebs solution employed contained (mM) Na^+ 136 9 K^+ 5 9 Ca^{++} 2 5 Mg^{++} 1 2 HCO_3 15 5 $H_2PO_4^-$ 1 2 Cl^- 133 6 and glucose 11 5 When the effects of varying $[K^+]_o$ or $[Ca^{++}]_o$ were studied the procedures were as follows

I *Change of $[K^+]_o$ from 5 9 mM to zero* The strips were equilibrated for one hour at 5 9 mM K^+ a dose response curve to phenylephrine or carbachol worked out the bathing fluid changed 2–3 times until the tension had fallen to the initial base-line $[K^+]_o$ was then changed to zero and the dose-response curve repeated after a new equilibration period of 40 min

II *Change of $[K^+]_o$ from 0 74 mM → 1 475 mM → 2 95 mM → 5 9 mM (1/8 – standard $[K^+]_o$)* One hour equilibration at 0 74 mM K^+ dose response curve wash out change of $[K^+]_o$ in test bath to 1 475 mM wait 20 min new dose response curve wash out change of $[K^+]_o$ to 2 95 mM, wait 20 min new dose response curve wash out etc

III *Change of $[K^+]_o$ from 5 9 mM → 11 8 mM → 23 6 mM → 47 2 mM → 94 4 mM (1–16 times standard $[K^+]$)* As II above only starting with $[K^+]_o$ = 5 9 mM and increasing stepwise to 94 4 mM

IV *Change of $[Ca^{++}]_o$ from 0.31 mM → 0 63 mM → 1 25 mM → 2.5 mM → 3 73 mM (1/8 3/2 times standard $[Ca^{++}]_o$)* Higher concentrations of Ca^{++} cannot be tested in the present solution at 38° as Ca^{++} precipitates when $[Ca^{++}]_o$ is increased further) As II above $[Ca^{++}]_o$ being changed in instead of $[K^+]_o$ in the test bath

V *Change of $[Ca^{++}]_o$ from 0.31 mM to zero* As I above

In series III $[Na^+]_o$ was reduced corresponding to the increase in $[K^+]_o$ Since the ionic alterations in the other experiments were very small the changes in ionic composition were made simply by altering the amount of isotonic KCl or $CaCl_2$ added to the Krebs solution

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III *Change of* $[K^+]_o$ *from 5 9 mM → 11 8 mM → 23 6 mM → 47.2 mM → 94 4 mM (1–16 times standard* $[K^+]_o$*)* As II above only starting with $[K^+]_o$ = 5 9 mM and increasing stepwise to 94 4 mM

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As seen from fig 1 the total change in response due to the alteration in $[K^+]$, can be calculated by subtracting the response in the test preparations (observed response) from the response in the controls (expected response) Expressed in per cent of the initial maximal contraction, the median reduction in maximal response produced by lowering $[K^+]_o$ from 5.9 mM to zero was 142.5 (control response) − 44.9 (test response) = 97.6 for phenylephrine and 120.9 − 77.8 = 43.1 for carbachol The difference in effect on phenylephrine and carbachol is of borderline significance (Wilcoxons two sample test, phenylephrine 8 preparations, carbachol 8 preparations $0.1 < P < 0.05$)

Change of $[K^+]_o$ from 0.74 mM to 5.9 mM, and from 5.9 mM to 94.4 mM

Fig 2 shows how the responses to phenylephrine 10^{-4} M and carbachol 2×10^{-5} M change with changing $[K^+]_o$ It will be seen that the responses to phenylephrine increases when $[K^+]_o$ is raised from 0 to 23.6 mM (statistical significance *0.74 → 5.9 mM*, 6 pairs tested, median increase 16 %, $P < 0.05$, *5.9 → 11.8 mM*, 8 pairs, median increase 10 % $P < 0.01$. Only

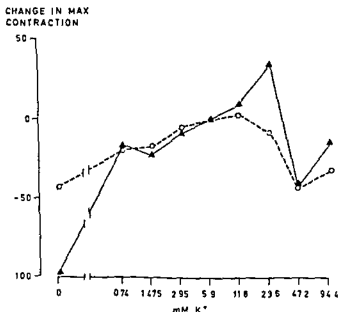


Fig 2 Contractile responses to phenylephrine 10^{-4} M and carbachol 2×10^{-5} M at varying $[K^+]_o$ (log scale) Ordinate Change in maximal contraction in the test preparations relative to the controls expressed in per cent of the maximal contraction in each preparation at $[K^+]_o = 5.9$ mM The response to phenylephrine is represented by filled triangles the response to carbachol by the open circles and dotted line (Median values of 4–8 strips for statistical evaluation see text)

4 pairs were tested at *23 6 mM*, and the median increase for these were 32 %, 0 15 $< P <$ 0 1), the maximal response falls when $[K^+]_o$ is increased further (8 pairs, median reduction 41 5 %, $P <$ 0 05)

The response to carbachol also increases with increasing $[K^+]_o$, *0 74 → 5 9 mM*, 6 pairs, median increase 20 %, $P <$ 0 05, *5 9 → 11 8 mM*, 6 pairs, median increase 0 2 %, $P =$ 0 5) but less pronounced than the maximal response to phenylephrine At $[K^+]_o$ of 47 2 and 94 4 mM the maximal response to carbachol was also significantly reduced (6 pairs, median reduction 44 9 and 31 7, $P <$ 0 05 for both) The differences between the effects on the responses to phenylephrine and carbachol at 23 6 mM is not statistically significant (Wilcoxon two sample test, phenylephrine 4 preparations, carbachol 6 preparations $P <$ 0 7)

The marked reduction in the contractile responses at the highest concentrations of K^+ is probably due to the depolarizing effect of K^+ In circular rabbit strips as in most other intestinal preparations these concentrations of K^+ produced a transient contraction which subsided long before the dose-response curves to phenylephrine and carbachol were carried out It should be noted that even at the highest concentrations of K^+, where the cell membranes probably are completely depolarized, typical dose response curves to both drugs could be worked out (*fig* 3) This *indicates* that the contractile effect of these drugs can be dissociated completely from the effect on the membrane potential

Change of $[Ca^{++}]_o$ from 0 31 mM to zero and from 0.31 mM to 3 75 mM

Alterations in $[C^{++}]_o$ produced changes in the dose response curves to phenylephrine and carbachol similar to those produced by alterations in $[K^+]_o$, i e marked changes in maximal response but no definite alteration in sensitivity

The changes in maximal response due to the alteration in $[Ca^{++}]_o$ have been plotted semi logarithmically in fig 4 The responses to both phenylephrine 10^{-4} M and carbachol 2×10^{-5} M increase with increasing $[Ca^{++}]_o$ throughout the range tested (0–3 75 mM) It should be noted that the increase is most pronounced for phenylephrine *$[Ca^{++}]_o$ = 0 31 mM → 0*, median reduction in response to phenylephrine 10^{-4} M in 6 pairs = 74 9 %, $P <$ 0 05, median reduction in response to 2×10^{-5} M carbachol in 6 pairs = 63 %, $P <$ 0 05, $[Ca^{++}]_o$ = *0 31 → 3 75 mM* median increase in response to phenylephrine 10^{-4} M in 6 pairs = 171 9 %, $P <$ 0 05, median increase in response to carbachol 2×10^{-5} M in 6 pairs = 50 4 %, $P <$ 0 05) This difference in effect on the responses to phenylephrine and carbachol is statistically significant (Wilcoxon's two sample test, phenylephrine 6 preparations, carbachol 6 preparations, $P <$ 0 05)

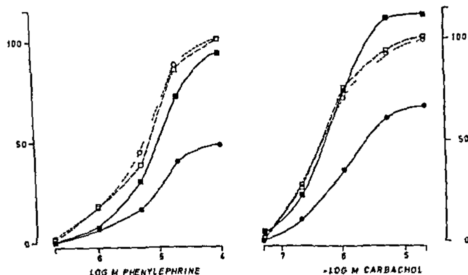


Fig 3 Effect of increasing $[K^+]_o$ from 5 9 to 47 2 mM The dotted lines (open squares and circles) show the initial log dose response curves at $[K^+]_o$ = 5 9 mM the continuous lines show how increase of $[K^+]_o$ to 47 2 mM alters the dose response curve for the test preparations (filled circles) compared with the dose response curve in the controls (filled squares) which has been repeated without altering $[K^+]_o$ (Phenylephrine median of 8 strips carbachol median of 6)

Discussion

The present study shows that the magnitudes of the responses to phenylephrine and carbachol depend both on the external potassium and the external calcium concentration The responses increased with increasing $[K^+]_o$ from zero to 23 8 mM and with increasing $[Ca^{++}]_o$ from zero to 3 75 mM but were reduced at $[K^+]_o$ of 47 6 and 94 4 mM

It is hardly surprising that increasing $[Ca^{++}]_o$ should increase maximal responses, as it is generally accepted that Ca^{++} plays a key role in the linkage between excitation and contraction (HURWITZ & JOINER 1969) and it is also likely that the action potential in smooth muscle cells is mainly dependent on Ca^{++}-fluxes (BULBRING & TOMITA 1970, NAGASAWA & SUZUKI 1970 KURIYAMA 1970)

The increase in response when $[K^+]_o$ is raised from zero to 23 6 mM is more difficult to explain Since increased permeability to K^+ seems to be a general effect of α adrenoceptor stimulation, regardless of whether the response is contraction or relaxation (DANIEL *et al* 1970) it seems unlikely that K^+ should have any direct influence on the contractile mechanism

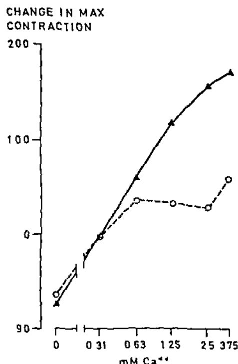


Fig 4 Contractile responses to phenylephrine 10^{-4} M and carbachol 2×10^{-5} M at varying $[Ca^{++}]_o$ (log scale) Ordinate Change in maximal contraction in the test preparations relative to the controls expressed in per cent of the maximal contraction in each preparation at $[Ca^{++}]_o = 0\,31$ mM The response to phenylephrine is represented by filled triangles the response to carbachol by open circles and dotted line (Median values of 6 strips)

The contractile response to pilocarpine in guinea-pig ileum certainly still occurs after the associated increase in K^+ efflux has been inhibited by deoxycorticosterone acetate (BASS *et al* 1964) This indicates that the increased permeability to K^+ is merely a secondary effect of excitation, and not a causal factor in the initiation of the mechanical response It seems most likely that the increased magnitude of the responses with increasing $[K^+]_o$ is due to a secondary effect on available Ca^{++} It may be that the concentration of K^+ on the outside of the cell membrane is important for the regulation of efflux of Ca^{++}, or it could be that Ca^{++} is less firmly bound to fixed anionic sites when there is more K^+ present which competes for these sites (GOODFORD 1965, 1966 & 1967)

The reduced magnitude of response at external potassium concentrations of 47 2 and 94 4 mM may be due to the marked depolarisation of the cell membrane which these high $[K^+]_o$ produce (CASTEELS & KURIYAMA 1966, CASTEELS 1970 KURIYAMA 1970) Although contractile responses can be

produced in depolarized muscle (AXELSON 1970), contractions are normally associated with transient or sustained depolarization and the abolition of this part of the response in muscle which is depolarized beforehand, may explain the reduction in response

In the present experiments it was noted that the response to phenylephrine and carbachol differed slightly in their relationship to $[K^+]_o$ and $[Ca^{++}]_o$ The reduction in maximal response to phenylephrine at zero $[K^+]_o$ was much greater than the reduction in the response to carbachol, and the increase in response to phenylephrine with increasing $[Ca^{++}]_o$ above 0 63 mM was much greater than the increase in the contractile response to carbachol It has been reported that the cellular mechanisms underlying the excitatory effects of noradrenaline, histamine and potassium in vascular smooth muscle differ Noradrenaline appears to be able to liberate firmly bound intracellular calcium, whereas the contractile effects of histamine and potassium are almost entirely dependent on free Ca^{++} (HUDGINS & WEISS 1968) The differences between the responses to phenylephrine and carbachol found in the present experiments may reflect similar differences in cellular mechanisms of action, carbachol may be more able to liberate bound calcium than phenylephrine since it produces a much greater maximal contraction than phenylephrine

In conclusion it may be said that the present series have shown that the magnitude of the contractile responses to phenylephrine and carbachol in circular rabbit fundus strips is dependent both on the external potassium concentration and the external calcium concentration, but that contractile responses to both drugs can be obtained in solutions which are depleted of K^+ or Ca^{++} The present experiments also indicate that there may be quantitative differences in the mechanisms underlying the contractile responses to phenylephrine and carbachol

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The Effect of Varying External K^+ and Ca^{++} on the Increase in ^{42}K-efflux Produced by Phenylephrine and Carbachol in Circular Rabbit Fundus Strips

By

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(Received February 7, 1973 Accepted February 14 1973)

Abstract Circular strips from the fundus of rabbit stomachs were incubated for one hour in radioactive (^{42}K) modified Krebs solution They were then transferred to a constant flow apparatus where they were washed with inactive Krebs solutions with K^+ concentration ($[K^+]_o$) of zero 5 9 (standard) 23 6 and 94 4 mM and with Ca^{++} free solution ($0[Ca^{++}]_o$) After an initial equilibration period of 32 min the strips were exposed to 10^{-4} M phenylephrine or carbachol The ^{42}K efflux the effects of the two drugs on ^{42}K efflux, and the mechanical responses were studied The ^{42}K efflux was lowest at 5 9 mM K^+, and highest at 94 4 Both phenylephrine and carbachol increased efflux at $[K^+]_o$ of zero and 5 9 mM and at $0[Ca^{++}]_o$ Carbachol also increased efflux at $[K^+]_o$ of 23 6 mM Mechanical responses to both drugs were obtained at all $[K^+]_o$ and at $0[Ca^{++}]_o$ Both the mechanical responses and the alterations in efflux were greatest at 5 9 mM K^+ but otherwise no correlation was found between the magnitude of these two types of responses As the incubation medium had been modified correspondingly to the washing fluid the K uptake at the various $[K^+]_o$ and at $0[Ca^{++}]$ could also be determined The K^+ uptake at $0[Ca^{++}]_o$ was 49 3 mM K^+/kg dry weight at $0[K^+]_o$ 64 9 mM/kg at standard $[K^+]_o$ 145 2 mM/kg at 23 6 mM K^+ 176 9 mM/kg and at 94 4 K^+ it was 890 1 mM/kg The uptake at standard $[K^+]_o$ was lower after cold storage for two days than after one day, but the length of storage did not appear to influence ^{42}K efflux or the responses to phenylephrine and carbachol

Key words K efflux K uptake – adrenergic receptors – cholinergic receptors – gastric smooth muscle – gastric motility

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We have previously reported that both cholinergic stimulation with carbachol and α adrenergic stimulation with adrenaline, noradrenaline or phenylephrine produce contraction in circular rabbit fundus strips, and that these contractile responses are assosiated with transient increases in ^{42}K efflux (HAFFNER *et al* 1972) In a recent investigation (HAFFNER 1973) it was found that the contractile responses to both phenylephrine and carbachol increased with increasing external Ca^{++} concentrations ($[Ca^{++}]_o$)

from zero to 3 75 mM, and with increasing external K^+-concentrations ($[K^+]_o$) from zero to 23 6 mM, but were reduced at higher $[K^+]_o$. The present study was undertaken in order to determine whether alterations in $[K^+]_o$ and $[Ca^{++}]_o$ had the same influence on the increases in ^{42}K-efflux produced by phenylephrine and carbachol

Materials and Methods

As described previously (HAFFNER et al 1972) circular rabbit fundus strips without mucosa were prepared from male white rabbits The strips were stored overnight at 4° On the next day they were incubated in aerated (95 % O 5 % CO) radioactive (^{42}K) Krebs solution at 38° for one hour, and mounted in a constant flow apparatus where they were washed continuously with warm (38°) oxygenated inactive Krebs solution which after passing through the apparatus was collected in test tubes and changed every two min As previously the radioactivity in the test tubes as well as that remaining in the strips at the end of the experiments was determined in a Packard Auto gamma spectrometer Model 2001 The drugs were added after a 32 min control period but in the present investigation the effluent solution from the constant flow apparatus was collected for only 16 min after addition of the drugs This time was more than ample to measure the transient increase in ^{42}K efflux produced by the drugs The Krebs solution used previously contained (mM) Na^+ 136 9 K^+ 5 9 Ca^{++} 2 5, Mg^{++} 1 2 HCO_3^- 15 5 $H_2PO_4^-$ 1 2 Cl^- 133 6 and glucose 11 5 (pH 7 4) This solution will be referred to as standard solution In the present experiments the standard solution was used with the following modifications

I *Experiments with* $0[K^+]_o$ Storage overnight and incubation (with ^{42}K) in standard solution Washing with standard solution without K^+

II *Experiments with* $4[K^+]_o$ Storage in standard solution incubation (with ^{42}K) and washing with $4[K]_o = 23.6$ mM

III *Experiments with* $16[K^+]_o$ Storage in standard solution Incubation and washing in $16[K^+]_o = 94.4$ mM
In both incubation and washing solutions the K^+ ions were replaced by a corresponding amount of Na^+

IV *Experiments with* $0[Ca^{++}]_o$ Storage incubation (with ^{42}K) and washing in standard solution completely depleted of calcium

The initial specific activity in the incubation medium was approximately 6 µci/ml in all experiments

The dry weight of the preparations was determined after at least 48 hrs drying at 60°

Drugs Carbacholine chloride (carbacholinum chloridum NFN) and phenylephrine HCl (metaoxedrinum NFN) were added to the washing solution in amounts which gave final concentrations of 10^{-4} M The drugs were prepared each day from frozen stock solutions Radioactive potassium (^{42}K) was supplied by Institutt for Atomenergi Kjeller, Norway

Calculations All values given are median values The content of ^{42}K in the preparation throughout the experiment, and the fraction lost were determined as described previously (HAFFNER et al 1972) The half time for K^+ efflux for the slow part of the efflux curves was calculated from the difference in K^+ efflux expressed as counts

min^{-2} at 20 and 40 min The effects of the drugs were recorded as the maximal efflux after addition of the drugs expressed as per cent of the efflux value on the last interval before the drug was added These values have been compared with corresponding values for controls which were washed out without addition of any drug Wilcoxon s two sample test was used for statistical evaluation (α denotes significance)

Results

K-efflux at varying $[K^+]_o$ and at $0[Ca^{++}]_o$ (controls)

In fig 1 fraction lost curves for ^{42}K at varying $[K^+]_o$ and at $0[Ca^{++}]_o$ are compared with the fraction lost curve at standard $[K^+]_o$ and $[Ca^{++}]_o$ obtained previously (HAFFNER *et al* 1972) The fraction lost per min. was lower in standard solution than at any other $[K^+]_o$ or $[Ca^{++}]_o$ The median values for fraction lost 32 min after the start of washing out are shown in table 1, which also shows the half times for the efflux curves The half time for efflux in standard solution was 43 1 min based on the reduction in K efflux from 20 to 40 min, 64 5 min when based on the reduction from 20 to 60 min (HAFFNER *et al* 1972) and 87 1 min when based on the reduction from 40 to 60 min

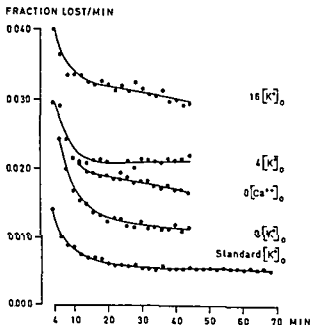


Fig 1 Fraction of ^{42}K lost per min at varying external K$^+$ concentration (standard $[K^+]_o$ = 5 9 mM $4[K^+]_o$ = 23 6 mM $16[K^+]_o$ = 94 4 mM $0[K^+]_o$ = K$^+$ free solution) and in Ca$^+$ free solution ($0[Ca^{++}]_o$) Median curves of 12–13 preparations

Table 1

K efflux at varying $[K^+]_o$ and at $0[Ca^{++}]_o$ *

	$0[Ca^{++}]_o$	Standard	$0[K^+]_o$	$4[K^+]_o$	$16[K^+]_o$
Fraction lost					
at 32 min	0 0181	0 0056	0 0116	0 0219***	0 0319***
α**	$<$0 0001		0 007	$<$0 0001	$<$0 0001
Half time					
(min)	23 9	43 1	31 8	32 2	18 3
α**	$<$0 0001		0 005	$<$0 0001	$<$0 0001

* Median of 12–13 preparations
** α = significance of difference from standard
*** The increase from $4[K^+]_o$ to $16[K^+_o]$ is also statistically significant $\alpha < 0\ 0001$

Effects of phenylephrine and carbachol on K efflux at varying $[K^+]_o$ and at $0[Ca^{++}]_o$

The changes in K efflux produced by phenylephrine and carbachol at varying $[K^+]_o$ and at $0[Ca^{++}]_o$ are presented in table 2 The increase in efflux was always transient and lasted for only 4–8 min

K efflux was significantly increased by phenylephrine in solutions with standard $[K^+]_o$, $0[K^+]_o$ and $0[Ca^{++}]_o$, but no increase was obtained in $4[K^+]_o$ and $16[K^+]_o$ No difference was found in the magnitudes of the increases at standard $[K^+]_o$ and $0[K^+]_o$ or between standard solutions and $0[Ca^{++}]_o$ Carbachol increased K-efflux significantly in all solutions except $16[K^+]_o$-

Table 2

K efflux after addition of phenylephrine or carbachol *

	$0[Ca^{++}]$	Standard	$0[K^+]_o$	$4[K^+]_o$	$16[K^+]_o$
Controls	89 71	97 80	94 89	91 19	92 23
Phenylephrine	101 2	109 1	103 5	91 0	91 6
α**	0 0003	0 002	0 01	–	–
Carbachol	110 9	142 9	109 7	112 8	96 2
α**	0 0005	0 0001	0 0006	0 0001	0 17

* Median values of 6–12 preparations K efflux is expressed in per cent of the existing efflux when the drug was added
** α = significance of difference from controls

solution The increase in ^{42}K-efflux produced by carbachol in standard solution was significantly greater than in any other solution ($0[K^+]_o$ $\alpha = 0.05$, $4[K^+]_o$ $\alpha = 0.02$, $0[Ca^{++}]_o$ $\alpha = 0.01$)

Contractile responses to phenylephrine and carbachol at varying $[K^+]_o$ and at $0[Ca^{++}]_o$

The contractile responses to phenylephrine and carbachol at varying $[K^+]_o$ and at $0[Ca^{++}]_o$ are presented in table 3 The contractions were maximal in standard solutions, just like the increases in K-efflux, but contractile responses were obtained at high $[K^+]_o$ when no significant increase in K-efflux occurred (phenylephrine $4[K^+]_o$ and $16[K^+]_o$, carbachol $16[K^+]_o$) No correlation was found between the contractile force developed at the various $[K^+]_o$ and $[Ca^{++}]_o$, and the increases in K-efflux (The contractile responses and increases in K-efflux produced by carbachol appeared to be correlated, but Kendall's correlation coefficient was not significantly different from zero, $\alpha \sim 0.2$)

Effects of varying storage time and incubation conditions on uptake of K^+

As $[K^+]_o$ and $[Ca^{++}]_o$ in the storage and incubation medium varied in the different test groups in the present investigation, it was found desirable to compare the uptake of K in the various groups The medium uptakes of K in the control preparation were standard solution 145.2 mM/kg dry weight, $0[K^+]_o$ 64.9 mM/kg, $4[K^+]_o$ 176.0 mM/kg($17[K^+]_o$ 890.1 mM/kg, $0[Ca^{++}]_o$ 49.3 mMkg The preparations tested in standard solution and at $0[K^+]_o$ had all been stored and incubated in standard solution, they therefore had to be considered as one group with regard to K-uptake The uptake of K at $0[Ca^{++}]_o$ was significantly lower ($\alpha < 0.0001$), and the uptakes at $4[K^+]_o$ and $16[K^+]_o$ significantly higher ($\alpha < 0.0001$), than at standard The difference between $4[K^+]_o$ and $16[K^+]_o$ was also significant ($\alpha < 0.0001$) Surprisingly, the difference between the preparations tested at standard and

Table 3

Contractile responses (mg) to phenylephrine and carbachol *

	$0[Ca^{++}]_o$	Standard	$0[K^+]_o$	$4[K^+]_o$	$16[K^+]_o$
Phenylephrine	250	2200	775	1140	890
α**	0.001		0.003	0.01	0.03
Carbachol	650	3375	1020	2200	2300
α**	0.0004		0.04	0.01	0.03

* Median values of 6–12 preparations, the same as in table 2

** α = significance of difference from standard

$0[K^+]_o$ was also found to be significant ($\alpha = 0\,007$) in spite of identical storage and incubation solutions Further analysis showed that the difference was due to different durations of storage Whereas 8 of the preparations tested at standard solution had been stored one day and 4 had been stored 2 days, only 3 of the preparations tested at $0[K^+]_o$ had been tested after one day's storage, the remaining 8 after two days When the uptake was related to storage, the median uptake was found to be 153 7 mM/kg after one day and 73 2 mM/kg after two days. The difference is statistically significant ($\alpha = 0\,004$) The length of storage did not appear to influence K-efflux or the responses to phenylephrine or carbachol

Discussion

The present study shows that K-efflux varies at different $[K^+]_o$ and that it is lowest at standard $[K^+]_o$ ($\alpha = 5\,9$ mM) Corresponding results have been obtained in studies of K-efflux from taenia coli preparations, both removal of external K^+ and increase in $[K^+]_o$ increased K-efflux (SETEKLEIV 1967, CASTEELS 1970) It has also been reported that Ca^{++} depletion may increase K-efflux from longitudinal ileum preparations (WEISS & HURWITZ 1963, VON HAGEN & HURWITZ 1967), as it did from rabbit fundus preparations in the present study In the taenia coli, however, depletion of Ca^{++} caused no change in K^+-efflux in standard Krebs solution (CASTEELS 1970)

Studies on ^{40}K-efflux from taenia coli and vas deferens preparations have shown that the efflux follows a single exponential time course during the first 150 min (CASTEELS 1970) Half times for the efflux from taenia coli have been reported as 68 min (CASTEELS 1970) and 75 min (BORN & BULBRING 1956) and from vas deferens 111 min (CASTEELS 1970) In our experiments there was an initial rapid wash-out of ^{40}K, probably reflecting extracellular potassium, but after 20 min the efflux seemed to follow a single exponential time course In the preparations studied at standard solu tion, which had been followed for 70 min, it was found out, however, that the K efflux slowed up with increasing time, the calculated half-time varying from 43 to 87 min depending on which time interval between 20 and 60 min had been used for the calculations The most likely explanation for this deviation from the single exponential function seems to be the variability in the operation of individual cells (VAN LIEW 1967)

In the present study phenylephrine caused an increase in K-efflux both in K^+ and Ca^{++} depleted solution, but not at $[K^+]_o$ of 23 6 or 94 4 mM This is in contrast to what other investigators have found in other types of smooth muscle Only small and inconsistent increases in K-efflux were obtained when the adrenergic α receptors in taenia coli and rat uterus preparations were stimulated in standard Krebs solutions, but more consistent increases

occurred after the tissues had been depolarized with K_2SO_4 rich solution (BUI BRING *et al* 1966, JENKINSON & MORTON 1967, DANIEL *et al* 1970) WAHLSTROM (1972) found that noradrenaline increased K-efflux (10–20 %) in rat portal vein in normal solution, whereas it had no effect on K-efflux in K^+-high and Ca^{++}-free solution

In the present investigation carbachol produced a significant increase in K-efflux in all solutions, except that containing 94 4 mM K^+. The lack of increase in potassium-rich solution is in disagreement with the results of DURBIN & JENKINSON (1961) who measured the effect of carbachol on ^{42}K efflux on muscle from the taenia coli depolarized in K_2SO_4-rich solution, and found that efflux was doubled in a period of 7 min after application of the drug. When the data from our experiments were treated in the same way as theirs, there was still no increase in K-efflux in our experiments

In our experiments the increase in efflux produced by carbachol in $0[Ca^{++}]_o$ solution, was smaller than that in normal solution WEISS & HURWITZ (1963) observed that in ileal smooth muscle acetylcholine gave a greater than normal increase in ^{42}K-efflux when the tissue had been incubated for 5 min in Ca-free solution

The increases in both K-efflux and contractile force were greatest in standard solution, but in agreement with our previous investigation (HAFFNER *et al* 1972) we failed to find any direct correlation between the effect on K-efflux and contractile force As pointed out previously these inconsistent findings could be due to the fact that the magnitude of K-efflux depends mainly on the total mass of the preparations, whereas the magnitude of the mechanical responses depends on the thickness and degree of stretch As discussed elsewhere (HAFFNER 1973) contractile responses to pilocarpine can still be obtained after the associated increase in K-efflux has been inhibited (BASS *et al* 1964) This is in accordance with the observation made in the present investigation, that contractile responses can be obtained at high $[K^+]_o$ without any increase in K-efflux As previously pointed out (HAFFNER 1973) this suggests that the increased permeability to K^+ is not a necessary step in the contractile response The drugs probably act directly on the contractile elements under these circumstances (SCHILD 1964)

In the present study it was also attempted to determine how varying $[K^+]_o$ and $[Ca^{++}]_o$ affected the uptake of K^+ into fundus strips It was found that the uptake was increased by increasing $[K^+]_o$, which is consistent with the findings of DANIEL *et al* (1970) on rat uterus As efflux also increased under these conditions, they seem to produce a general increase in K permeability Removal of Ca^{++} on the other hand, increased efflux but reduced uptake These findings would be consistent with a hypothesis of a linkage between Ca ions at the cell membrane and the Na/K pump, a reduction of Ca ions leading to diminished work of the pump

The duration of cold storage appeared to affect uptake and efflux in different ways, the uptake after two days cold storage was less than after one, but no effect on efflux was found, either in the controls, or in the responses to phenylephrine or carbachol As the incubation period was quite long (one hour), it seems unlikely that the effect of cold storage on efflux should differ merely because the preparations had not had time to recover before uptake and efflux were tested Using taenia coli (BAUER *et al* 1963) it has been shown that cold storage up to 24 hours does not affect the uptake of ^{42}K in one hour The changes seen in our experiments on the rate of uptake, are probably due to the effect of cold storage on the mechanisms which regulate influx, and must have occurred during the last 24 hours of storage

In conclusion it can be said that the present investigation has shown that varying $[K^+]_o$ and $0[Ca^{++}]_o$ increase K-efflux and modify the effect of phenylephrine and carbachol on both K-efflux and contractile force It also confirms our previous suggestion that there is no direct correlation between the alteration in K efflux and contractile response to phenylephrine or carbachol

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The Haemolytic Properties of the Oriental Hornet Venom

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Abstract Venom from *Vespa orientalis* haemolyses erythrocytes of man, guinea pig, rabbit cat, mouse and rat, but not of sheep, ox, horse and camel The haemolytic effect was enhanced by the addition of egg yolk phospholipids Boiling destroyed the haemolytic factor(s), but the haemolytic activity which was demonstrable after addition of phospholipids resisted boiling at pH 3 5 The haemolytic factor(s) could be removed by heparin precipitation and Amberlite adsorption and could be neutralized by rabbit anti wasp venom serum The haemolytic factors could not be removed by dialysis Sublytic doses of whole venom caused potassium leakage from susceptible erythrocytes and increased their osmotic fragility It is suggested that the indirect haemolytic activity of the Oriental hornet s venom is associated with phospholipase activity, while the direct lytic activity is due to basic protein

Key words Haemolysis – Oriental hornet venom

The haemolytic activity of animal toxins has been extensively studied and a large literature exists on the subject, based both on clinical and laboratory observations (DACIE 1967) Most of the studies concern the *in vivo* and *in vitro* haemolytic effects of snake and bee venom (ROY 1945, GITTER *et al* 1959, KLIBANSKY & DE VRIES 1963, CONDREA *et al* 1964b, HABERMANN 1968) Wasp venoms have been relatively little studied, due to difficulties in obtaining their venoms in sufficient amount The available information concerns the common wasp *Vespa vulgaris* (JACQUES & SCHACHTER 1954, SCHACHTER & THAIN 1954), the hornet – *Vespa crabro* (NEUMANN & HABERMANN 1956, BHOOLA *et al* 1961) and the Oriental hornet – *Vespa orientalis* (ISHAY *et al.* 1970, 1971 & 1972, EDERY *et al* 1972) Clinical observation of renal damage following wasp stings (JONAS & SHUGAR 1964) and renal changes after envenomation of mice (SANDBANK *et al* 1972) have been described Recently, the presence of haemolytic fractions in the venom of

the Oriental hornet has been reported (JOSHUA *et al* 1971, FISCHL *et al.* 1972)

In this report an investigation of the haemolytic properties of the Oriental hornet venom is presented

Materials and methods

Hornet venom was obtained in the native form from live insects, using the following technique the abdomen of the insect was held with forceps and gentle pressure was applied until a small drop of venom appeared at the end of the protruding sting It was carefully collected with a fine glass capillary Venom obtained in this way from many insects was pooled lyophylised and kept at −20° Before use, the venom was dissolved in saline to a protein concentration of 500 mg %, estimated by the method of Lowry

Blood was collected in 1/20 volume of 3 8 % sodium citrate from the following species man, ox, horse, camel, cat, sheep, rabbit, guinea pig mouse and rat The blood samples were washed 3 times in phosphate buffered saline – pH 7 0 and finally re suspended in a 5 % concentration v/v Egg yolk phospholipid emulsion was prepared by mixing 0 5 g egg yolk in 100 ml buffered saline containing $CaCl_2$ in a 0 05 M concentration The lytic activity of the venom was studied at various pH values ranging from 6 0 to 8 0 at intervals of 0 2 pH units

Acidification of the venom to pH 3 5 was done with 1/10 N HCl Heat inactivation of the venom was achieved in a boiling water bath for periods of 2 to 10 minutes Volume changes due to evaporation were estimated by weighing the tubes with the venom solutions before and after boiling and corrected by addition of saline Heparin precipitation of the direct lytic factor was done by adding gradually to the venom a solution of thromboliquine® (heparin sodium, Organon) containing 50 mg/ml heparin using a Hamilton micro syringe The heparin treated venom was then centrifugated at 3,500 r p m and the supernatant tested for residual haemolytic activity The sediment was washed in buffered saline and eluted with a veronal buffer pH 8 8, ionic strength 0 05 M by shaking for 30 min at 37° The direct lytic factor was adsorbed to Amberlite GC 50/200 mesh by shaking 1 ml of the whole venom with 250 mg of the resin powder, previously equilibrated with a 0 05 M sodium citrate/citric acid buffer pH 6 0

The haemolytic activity of the various venom preparations was tested by incubating known amounts of venom with aliquots of 0 5 ml erythrocyte suspensions Indirect haemolytic activity was measured by adding 0 1 ml of the egg yolk emulsion to the incubation mixtures All tubes were incubated for 2 hours at 37°, after which saline was added up to a final volume of 2 ml, the tubes were immediately centrifugated and the haemoglobin content of the supernatants was read in a Coleman Spectrophotometer at 576 mμ wave length The extent of the haemolysis was expressed as percentage of the total haemolysis obtained by addition of a minute amount of saponin powder

Osmotic fragility of human erythrocytes (DACIE 1963) was tested after incubation for 60 minutes at 37° with a sublytic amount of venom (0 5 μl/ml) Potassium leakage was studied by incubation at various intervals the RBC of different species with a sublytic dose (0 5 μg/ml of venom At the end of each period the tubes were centri fuged and the potassium content of the supernatants was determined using a flame photometer The values were expressed in meq /l of packed red cells, calculated from the packed volume of the erythrocytes used in the test

The lytic activity was studied at 0°, 20° and 37°

As the optical density of a haemoglobin solution at 620 mμ is practically nil while a suspension of erythrocytes gives a measurable reading in optical density units due to its turbidity, the rate of the reaction could be registered in the following way 1 8 ml of a 5 % suspension of human RBC in buffered saline was delivered in the cuvette of a Coleman Junior Spectrophotometer and the wave length of the instrument was set at 620 mμ 0 2 ml of a 1 % saponin solution was then added and the instrument was set at a 0 reading The cuvette was then thoroughly rinsed and dried 1 8 ml of the same erythrocyte suspension was again delivered with the addition of 0 2 ml saline and the optical density was recorded The cuvette was again cleaned and filled with 1 8 ml of erythrocyte suspension to which 0 2 ml of venom was added and rapidly mixed A stopper was set at the time of the introduction of the venom The needle of the instrument started to record a gradual fall in the optical density The times of halving of the optical density value were recorded and the procedure was followed until the reading fell almost to zero The percent of haemolysis was then calculated for each reading and the values plotted against the time of incubation

Antiserum against wasp venom was prepared by immunising rabbits as follows 10 μg venom with Freund s adjuvant were injected into the foot pad of the animals twice weekly for 5 weeks and a final booster dose of 100 μg was given intraperitoneally The rabbits were finally bled by cardiac puncture and the immune serum kept at −20° until used

For the neutralisation experiments aliquots of 0 1 ml serum were incubated for 15 min at 37° with various amounts of venom dissolved in 0 1 ml saline Thereafter 0 5 ml of a 5 % suspension of washed human erythrocytes was added and the mixtures were further incubated for 2 hours and then examined for the presence of haemolysis

Results

Whole venom of the Oriental hornet had a direct lytic activity detectable on washed human, guinea pig, cat, rabbit, rat and mouse erythrocytes No significant haemolysis was noted against horse, ox, camel and sheep erythrocytes at the highest concentration used (fig 1) The dose dependence followed a sigmoid curve with a relatively steep slope The haemolysis started at a venom concentration of 0 75 μg/ml or 1 50 μg/ml whole venom and was almost completed at concentrations ranging between 3 and 6 μg/ml

Incubation of human erythrocytes with whole venom in the presence of egg yolk phospholipids showed a much stronger haemolysis as compared to the degree of haemolysis in the absence of phospholipids This was observed both in terms of per cent haemolysis for a fixed period of incubation (table 1) as well as in terms of incubation time elapsed until a 100 % haemolysis of the test systems occurred A similar effect was also observed with regard to erythrocytes of guinea pig, rabbit, rat and mouse Incubation of erythrocyte suspension with haemolytic amounts of venom at 0°, yielded a negligible degree of haemolysis which fell to 2 % of the value recorded at 37° Incubation at room temperature decreased by only 10 % the degree of

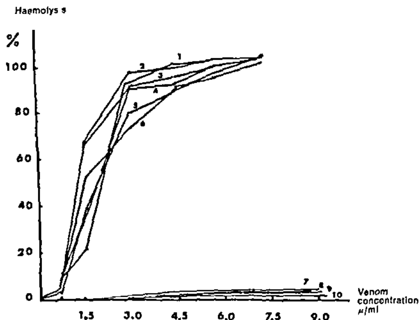


Fig 1 Effect of *Vespa orientalis* venom concentration on the degree of haemolysis of various erythrocytes 10 % erythrocytes suspension, 120 min incubation at 37° Types of erythrocytes used 1 – human 2 – guinea pig 3 – rat, 4 – mouse, 5 – rabbit, 6 – cat, 7 – ox, 8 – sheep 9 – horse, 10 – camel

haemolysis Investigation of the influence of the pH of the incubation mixtures showed a mild decrease in the haemolysis on the acid side with an optimum between pH 7 and 7.4 (table 2) The time kinetics of the

Table 1

Lytic effect of *V orientalis* venom on human RBC in the presence and in the absence of egg yolk phospholipids

Venom concentration µg/ml	% haemolysis without PL	% haemolysis with PL	Time of complete haemolysis (in min) without PL	Time of complete haemolysis (in min) with PL
0 75	50	90	208	143
1 50	88	100	145	102
3 00	100	100	58	31
6 00	100	100	25	13

The mixtures consisted of 0 5 ml of a 5 % erythrocyte suspension 0 1 ml venom, and 0 1 ml 0.5 % egg yolk phospholipids emulsion or 0 1 ml saline respectively All figures represent mean values of 6 consecutive determinations

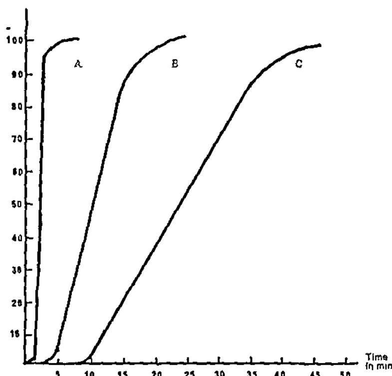


Fig 2 Time kinetics of the haemolysis of washed human erythrocytes threated with different concentrations of whole venom A – 6 µg/ml, B – 3 0 µg/ml, C – 1 5 µg/ml

haemolysis caused by whole venom is illustrated in fig 2 It can be seen that the haemolysis starts after a lag period the duration of which depends on the concentration of the venom used The curves obtained by plotting percent haemolysis versus time have a sigmoid shape and the steepness of the slope depends on the venom concentration In all the concentrations studied, the haemolysis slowed down after reaching a value of 90 to 95 %

Table 2

Influence of pH on the haemolysis of human RBC by the *V orientalis* venom

pH	6 0	6 2	6 4	6 6	6 8	7 0	7 2	7 4	7 6	7 8	8 0
Time of complete haemolysis* (min)	19	17	17	16	15	14	15	15	16	16	16

* Venom concentration 6 µg/ml

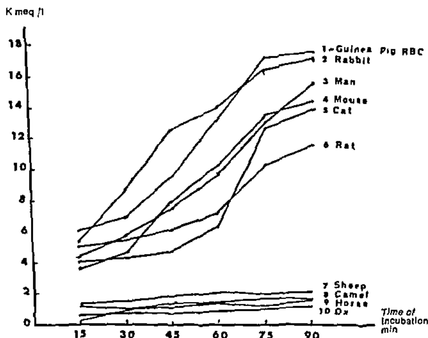


Fig 3 Potassium leakage from erythrocytes of different species incubated with a sub lytic dose (0 5 µg/ml) of *Vespa orientalis* venom

Table 3

Effect of various treatments on the haemolytic activity of *V orientalis* venom

Materials*	% Haemolysis** without PL***	% Haemolysis** with PL
Native venom	80	100
Venom heated at 100° (pH 6 0 or 8 0)		
for 2 min.	5	15
for 10 min	–	5
Venom heated at 100° – pH 3.5		
for 10 min	–	25
Venom after removal of heparin precipitable matter	5	35
i d heated for 2 min at 100° pH 6 0	–	22
Venom after Amberlite adsoption	–	28
Heparin precipitate	70	85
Heparin precipitate heated 2 min at 100°, pH 6 0	–	10

* equivalent to 3 µg/ml venom
** incubation at 37° for 120 min
*** Egg yolk phospholipid – 0 5 mg/ml

Pre incubation of human RBC with a sublytic concentration of whole venom (0 5 μg/ml) for 30 minutes at 37° resulted in an increase of their osmotic fragility, which started at 0 7 % NaCl and ended at 0 45 % NaCl At the same sublytic concentration, a variable degree of potassium leakage was found (fig 3) in accordance with the species susceptibility of the erythrocytes used As can be seen, human, guinea pig, mouse and rat erythrocytes which are susceptible to the lytic effect of *Vespa orientalis* venom showed a higher K leakage as compared to that of horse and ox erythrocytes which were previously found to be resistant to the lytic effect of the venom

Table 3 summarises the effect of dialysis as well as heat, heparin or Amberlite treatment on the haemolytic properties of the whole venom or the venom fractions As can be seen, heating the whole venom from 2–10 minutes in a boiling water bath at a pH of 6 0 or 8 0 caused the complete disappearance of the direct lytic effect on washed RBC even after 3 hours incubation There was also a considerable loss of the lytic activity in the presence of egg yolk phospholipids, which could be minimized if the venom was boiled at pH 3 5

The addition of heparin produced a fine precipitate which was separated by centrifugation for 30 min at 3,500 r p m The amount of heparin required to precipitate the basic protein component was approximately half of the venom total protein Both the heparin supernatant and the Amberlite supernatant were almost devoid of direct lytic activity, yet retained the indirect lytic activity after the addition of egg yolk phospholipids Dissociation of the heparin complex in veronal buffer containing an excess of heparin released a measurable lytic activity when tested on washed human RBC This activity was only mildly potentiated by the addition of egg yolk

Treatment with heparin of Amberlite likewise showed a clear cut decrease in the direct lytic effect with preservation of the indirect effect In all instances after elimination of the direct lytic factor the residual haemolytic activity of washed erythrocytes was extremely low The lytic activity in the

Table 4

Neutralisation of the haemolytic effect of whole venom of *V orientalis* by rabbit anti wasp venom immune serum*

Venom	Concentration (μg/ml) 384	192	96	48	24	12	6
Haemolysis	+	+	+	+	–	–	–

* 0 1 ml of rabbit anti wasp venom immune serum
\+ haemolysis
– absence of haemolysis

presence of phospholipids was lower than the original lytic activity of the total venom Continued dialysis of whole venom against saline at 4° did not cause any appreciable loss of the direct lytic activity Addition of rabbit anti whole venom immune serum to the whole venom, *in vitro* caused complete inhibition of the lytic activity (table 4) The lytic activity of wasp venom could be exhausted by repeated incubation with osmotically prepared and washed human erythrocyte ghosts The sedimented and washed ghosts were able to haemolyse a 5 % suspension of fresh human erythrocytes The haemolytic activity associated with the venom incubated ghosts was abolished by boiling at 100° and mildly potentiated by the addition of phospholipids

In a preliminary investigation on the *in vivo* haemolytic effect of *Vespa orientalis* venom using intravenous administration of a sublethal dose of whole venom to albino mice (2–2 5 mg per mouse weighing 20 g) free haemoglobin in the plasma and haemoglobinuria were found

Discussion

The results of the present study point toward a haemolytic system consisting of a direct lytic factor precipitable by heparin, probably a basic protein, and an indirect lytic factor, manifesting its activity in the presence of egg yolk phospholipids, and hence presumably a phospholipase A similar mechanism of haemolysis has already been described with regard to some snake venoms (ROY 1945, CONDREA *et al* 1964a) as well as to the honey bee venom (HABERMANN 1958) In all these venoms, the indirect haemolytic activity was attributed mainly to phospholipase A, which has no hydrolytic activity on the phospholipids of washed erythrocytes, yet could cause haemolysis in the presence of extracorpuscular phospholipids, originating from the plasma or even better from added egg yolk On the other hand, the direct lytic factors (DLF) from snake venoms or the melittin of honey bees venom are capable of lysing susceptible erythrocytes in the absence of external phospholipids Moreover, the presence of both factors results in a much stronger haemolytic effect, suggesting that the direct lytic factors, by changing the structure of the erythrocyte membrane, allow for the simultaneous hydrolysis of its phospholipids by phospholipase A Although the haemolytic activity of whole venom on washed erythrocytes reflects the combined effect of both the direct and the indirect factors, the presence of extracorpuscular phospholipids, which play a part in the formation of additional lysolecithin and free fatty acids, increase still further the extent of the haemolysis

The enhancing effect of egg yolk phospholipids is of particular significance in the present study, since up to the present we have been unable to

obtain purified fractions with exclusively direct or indirect haemolytic properties Therefore, the method allowed for the discrimination between the direct and the indirect haemolytic effect, the latter being defined as the increment of haemolysis obtained in the presence of extracorpuscular phospholipids

Boiling of the venom was also helpful in discriminating between the direct lytic activity which was completely lost and the indirect one which could be preserved to some extent at an acid pH The direct lytic factor was found in the heparin precipitate The activity of this fraction could also be destroyed by heat As the direct lytic factor could be adsorbed to Amberlite, it seems to be basic in nature

The direct lytic factor which seems to be firmly attached to the erythrocyte membrane of osmotic ghosts, cannot be easily removed by saline washing and retains its haemolytic properties toward freshly added erythrocytes Whether this is due to additional free active groups of the direct factor already attached to the ghosts, or to transfer of the direct factor from erythrocyte to erythrocyte is still a matter of conjecture Furthermore, the mildly potentiating effect of added egg yolk suggests that minute amounts of phospholipase may also have been attached to the osmotic ghosts

The differences in species susceptibility toward the haemolytic effect of *Vespa orientalis* venom are strikingly similar to those found with some snake lytic venoms (CONDREA *et al* 1964b) The explanation offered is based on the lecithin content of the red cells membranes, the erythrocytes which are poor in lecithin like those of the herbivores being resistant to haemolysis Furthermore additional factors concerning the membrane ultrastructure might also play a role, since the erythrocytes of the camel do not haemolyse although their membranes are quite rich in lecithin

The enhancing effect of egg yolk phospholipids was greatly reduced although still present when tested on isolated and washed heparin precipitate This finding might be due to contamination with traces of phospholipase but it could also be due to a complex formation of phospholipase with the direct lytic factor on the basis of ion exchange That basic proteins like melittin can aggregate in micelles of various size has already been shown (FISHER & NEUMANN 1961) thus accounting for the unhomogenous separation of melittin on moving boundaries electrophoresis Such a property might be responsible for the presence of multiple chromatographic fractions with direct haemolytic activity and is in agreement with the findings of FISCHL *et al* (1972) using electrophoretic separation of the haemolytic fractions

The potassium leakage and the "osmotic" type of haemolysis caused by exposing erythrocytees of susceptible species to sublytic concentrations of whole venom are reminiscent of the effect of lysolecithin, the haemolytic lysocompound generated from lecithin by phospholipase A

The explanation for the sigmoid shaped curve may be that the haemolytic system in fact consists of several factors with different kinetic characteristics

There are several similarities in common with the properties of the venoms from other wasps and hornets A direct lytic factor has been isolated from the venom of *Vespa vulgaris* (JAQUES & SCHACHTER 1954) which is precipitable by picric acid Curiously enough, the direct lytic factor of the picric acid precipitate is destroyed by boiling, but partly restored after acidification A similarly heat labile direct lytic factor has been found in the venom of *Vespa crabro* (HABERMANN 1968) Both phospholipase A and phospholipase B have been found in the venom of *Vespa vulgaris* (CONTARDI & LATZER 1928), as well as in the venom of *Vespa crabro* (HABERMANN 1968)

No clearcut answer can be given for the specificity of the antibody responsible for the *in vitro* neutralisation of the haemolytic activity by the rabbit anti wasp venom serum All we can say is that it completely neutralises the direct haemolytic activity of the whole venom Most of the immunisation experiments with animal venoms have mainly succeeded in the production of antiphospholipase antibodies (CINADER 1957, HABERMANN 1968), while melittin has proved a poor antigen In the case of the Oriental hornet's venom, however, the basic protein, being non dialysable, is presumably a large molecule with good antigenic properties

Although at present we have gained a better insight into the mechanism of the haemolysis caused *in vitro* by the venom of the Oriental hornet, there are still many questions awaiting elucidation The scarcity of the venom has so far prevented the use of more detailed procedures of purification and biochemical characterisation of the various venom fractions, which it is hoped will be achieved in the future

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Absence of Effects of Methysergide on Connective Tissue in Mice

By

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Abstract The effects of methysergide on the composition of skin liver and retroperitoneal tissue have been examined in mice After 14 days of treatment, no changes could be demonstrated in the contents of water, fat, fat free solids or hydroxyproline as compared with a control group nor was the plasma hydroxyproline level altered

Key words Methysergide – retroperitoneal fibrosis – hydroxyproline

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Reports have appeared since 1964 on a total of 70–80 cases of retroperitoneal fibrosis in migraine patients under treatment with methysergide (deseril®, sansert®) (GRAHAM 1964, GRAHAM *et al* 1966, SUBY *et al* 1965, UTZ *et al* 1965)

As remission of the disease is seen on cessation of the treatment (GRAHAM *et al* 1966, WEISS & HINMANN 1966, LINDENEG & KOK-JENSEN 1968) a causal relationship would seem probable Methysergide is also reported to have caused fibrosis of other organs, amongst others the lungs, pleurae, aorta and endocardium (GRAHAM 1967) so that the term *fibrotic* or *fibroplastic* effect has been used in connection with this drug In agreement with this the pathological process consists of an inflammatory response of unspecific type (GRAHAM *et al* 1966) However, knowledge is lacking as to how the drug produces this effect, in addition it is not clear, whether the changes occur only in particularly responsive individuals Animal experiments aimed at throwing light on this problem do not appear to have been carried out

The object of the present study has been to attempt to demonstrate any possible general "fibroplastic" effect of methysergide on the connective tissue The effects of the drug on the composition of the skin, liver and

the retro-peritoneal connective tissue have been examined in mice Particular emphasis has been given to the hydroxyproline contents of the organs as a measure of their collagen contents and to the hydroxyproline concentration of the plasma as a sensitive indicator of acute changes taking place in the connective tissue organs (LeRoy & Sjoerdsma 1965)

Methods

White male mice (Leo Stritt) weighing 25 30 g were divided into 2 groups One group (23 mice) was treated daily with methysergide (deseril® sansert®) 20 mg per kilo or 0.5 mg per animal in 0 5 ml fumerate buffer given intraperitoneally for 14 days The animals in the control group (19 mice) were injected with the same amount of buffer solution without methysergide Twenty four hours after the last injection the animal was stunned by a blow on the neck and 500 μl of blood sampled by severing the blood vessels on one side of the neck after which the animal was bled by decapitation A defined area of the depilated skin of the back including the subcutaneous tissue was removed the liver extirpated *in toto* and the retro-peritoneal connective tissue on the right side including tissue from the lower pre lumbar area removed as completely as possible The percentage contents of water fat and fat free dry substance of the skin liver and retro-peritoneal connective tissue were estimated by freeze drying and removal of the fat with ether and petrol/ether The hydroxyproline content was thereafter estimated on 5 mg of the dry fat free skin and 30 mg of the dry fat free liver according to Neuman & Logan s method in Martin & Axelrod s (1953) modification The fat free solids of the retro-peritoneal connective tissue constituted only approximately 1 mg per animal and hence the tissue was pooled in groups of 4–5 for the hydroxyproline estimation The total content of hydroxyproline in the plasma was estimated by a modification of the method described by LeRoy *et al* (1964) on 100–200 μl of plasma (cf Langgard 1968)

Results

Treatment with methysergide did not result in any change in the weight of the animals in relation to the control group The average weight changes during the test period in the treated group were 0 3 g (from −3 to +3 g) in the control group 0 0 g (from −2 to +3 g) There were no gross changes in the retro-peritoneal tissue

It can be seen from the table that the weight of the liver and the amount of retroperitoneal connective tissue did not alter during the treatment Also that the percentage contents of water, fat and fat free dry substance and of hydroxyproline in the skin, liver and retroperitoneal tissue were not effected Finally, that the concentration of hydroxyproline in the plasma was the same in the two groups

The figures in the table indicate mean values ± standard error of the mean

Table I

		Methysergide (n = 23)	Control (n = 19)
Skin	Water (%)	69 ± 0 5	66 ± 1 2
	Fat (%)	6 ± 0 6	9 ± 1 6
	Dried defatted weight (%)	25 ± 0 2	25 ± 0 5
	Water (g/100 g*)	271 ± 2 1	267 ± 4 4
	Hydroxyproline (g/100 g*)	9 0± 0 11	8 9± 0 14
Liver	Wet weight (mg)	1378 ± 31 0	1364 ± 41 6
	Water (%)	70 ± 0 4	71 ± 0 2
	Fat (%)	2 ± 0 1	2 ± 0 1
	Dried defatted weight (%)	28 ± 0.2	27 ± 0 4
	Water (g/100 g*)	257 ± 2 6	259 ± 2 4
	Hydroxyproline (mg/100 g*)	103 ± 5.5	116 ± 9 1
Retroperitoneal Connective Tissue	Wet weight (mg)	44 ± 4.3	55 ± 6 8
	Water (%)	28 ± 1 2	27 ± 1.5
	Fat (%)	3 ± 0.3	3 ± 0 5
	Dried defatted weight (%)	69 ± 1 4	70 ± 1 9
	Water (g/l g*)	10 ± 0 8	11 ± 0 9
	Hydroxyproline (g/100 g*)	1 1± 0 0**	1 3± 0 0**
Plasma	Hydroxyproline (mg/ml)	12 9± 0 41	12 4± 0 38

n = number of animals
* per 100 g dry defatted tissue
** n = 5

Discussion

Fibrosis is the final phase of any inflammatory reaction Biochemically this phase is characterized by a reduction in the water content and an increase in the collagen content of the tissue If treatment with methysergide, under the experimental circumstances described, exerted a fibrositic effect, it might be possible to demonstrate these changes in the organs, irrespective of whether the effect was due to fibroplast proliferation, liberation of biogenic amines, primary changes in the collagen molecules or to the anti serotonin effect of the compound (LEROY *et al* 1964)

The entirely negative results of this study are, of course, inconclusive, but are against a general connective tissue effect of methysergide Indirectly the results thus support the assumption that the development of fibrosis, observed at times in migraine patients under treatment with methysergide, is a singular manifestation of a variety of agents occurring in certain "fibrotically inclined individuals only

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Interspecies Variations in Small Intestinal and Hepatic Drug Hydroxylation and Glucuronidation

By

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(Received September 26 1972 Accepted December 13, 1972)

Abstract In order to study the interspecies variations in small intestinal and hepatic drug metabolism, the benzpyrene hydroxylase and UDP glucuronyl transferase activities were determined in the cat guinea pig mouse, rabbit and rat The cat exhibited very low benzpyrene hydroxylase and UDP glucuronyl tranferase activities both in its liver and small intestine. Of the other species the guinea pig had the highest benzpyrene hydroxylase activity, the rabbit an intermediate and the rat and mouse the lowest activity in the small intestine The hydroxylation of 3 4 benzpyrene by liver microsomes was most active in the mouse and guinea pig the rat liver showed an intermediate activity and the rabbit liver was least active The UDP glucuronyltransferase activity was highest in the small intestinal mucosa of the rabbit and rat, whereas the guinea pig and mouse exhibited an 80 per cent lower activity The guinea pig liver microsomes showed the highest activity and the mouse rabbit and rat livers had an intermediate activity The rat small intestinal benzpyrene hydroxylase activity could be enhanced to the same level as in the guinea pig by feeding the rats with a guinea pig diet

Key words Animals, laboratory – 3 4 benzpyrene – glucuronides – intestine small – liver – mucous membrane

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The testing of drugs and other chemicals for their therapeutic safety is carried out mainly on the common laboratory animals The possibility of species differences must always be considered in interpreting and extrapolating these results to man Thus the knowledge about the interspecies variations can aid in the design of pharmacological and toxicological studies in animals At present, it is well established that there are great differences in the susceptibility to foreign compounds and also in the metabolic fate of drugs in different animal species, although the knowledge of comparative pharmacology and comparative drug metabolism is, as yet, limited Particularly the hepatic drug metabolism has been rather extensively studied (for

reviews see WILLIAMS 1967, CONNEY 1967, PARKE 1968, SMITH 1968) Some species variations both in the intestinal drug oxidation and conjugation have also been described in separate studies (HARTIALA 1955, STEVENSON & DUTTON 1962, WATTENBERG *et al* 1962) The distribution patterns of the drug metabolizing enzymes in the gastro-intestinal tract are, however, rather poorly known

The aim of the present report has been to compare the activity of the drug oxidation and glucuronic acid conjugation and the distribution of the respective enzymes in the small intestinal mucosa and liver of the common laboratory animal species The hydroxylation of 3,4 benzpyrene and glucuronidation of *p* nitrophenol have been determined in the mucosal homogenates and liver microsomes Furthermore the effect of different diets has been studied by feeding rats with the pelleted diet of other species

Methods

Adult male Wistar rats (28) male rabbits (5) male mice (8) mongrel male cats (4) and guinea pigs (8) of both sexes were used The mice and rats were fed *ad libitum* on the same commercial mice pellets (HANKKIJA Oy Finland) as their sole food supply Both the guinea pigs and rabbits received rabbit pellets (HANKKIJA OY) but they were also supplied with bread and swedes In order to clarify the effect of different diets on the drug metabolism rat groups were fed for 9 days on the rabbit pellets and on the dog pellets A group of rats was also fasted for 72 hours before sacrifice The cats were fed on milk fish and meat

The animals were stunned by a blow on the head and bled by cutting the renal vessels The liver and small intestine were dissected at 4° and either 5 or 10 cm segments of the gut were cut out The mucosa was scraped with an ampoule file and homogenized with an Ultra Turrax homogenizer in one per cent digitonin solution as described earlier for the UDP glucuronyltransferase determinations (HANNINEN *et al* 1968) The mucosal samples for the benzpyrene hydroxylase determination were homogenized with a Potter Elvehjem type homogenizer with a Teflon pestle in 0 25 mol/l sucrose The liver microsomal fraction was sedimented by centrifugation at 105 000 × g (60 min at 0°) in 0 25 mol/l sucrose after presedimentation of the cell debris nuclei and mitochondria (VAINIO & HANNINEN 1972) The sediment was resuspended in 0 25 mol/l sucrose to yield a concentration of 1 g liver fresh weight/ml The microsomal protein content was determined by the biuret method (GORNALL *et al* 1948) The UDP glucuronyltransferase activity was determined as described by HANNINEN *et al* (1968) using *p* nitrophenol as aglycone and an UDPglucuronic acid concentration of 4 7 mmol/l The pH was 7 0 the incubation time of reaction varied according to the enzymatic activity between 10 and 20 minutes in order to work within the linear range of the reaction The 3 4 benzpyrene hydroxylase activity was measured as described earlier by GNOSSPELIUS *et al* (1969/1970) using about 0 1–0 2 mg of liver microsomes and mucosal homogenate equivalent to 20–40 mg of fresh mucosal weight. The UDP glucuronic acid and 3 4-benzpyrene were purchased from the Sigma Chemical Company (St Louis) and the *p* nitrophenol from E Merck AG (Darmstadt) Student s T test was used to calculate the statistical significances of the results

Results

The benzpyrene hydroxylase had very different distribution patterns in the small intestine of the various species Rats and mice had equally high activity in the duodenum, from which it decreased to the ileocaecal valve (fig 1A) In the small intestine of the guinea pig the mucosal benzpyrene hydroxylase activity increased from the pylorus to the end of the duodenum reaching a plateau in the jejunum and ileum, while the activity decreased again at the end of the ileum (fig 1B) The benzpyrene hydroxylase activity was ten times higher in the guinea pig than in the rat and mouse duodenum The rabbit showed an aborally increasing activity of benzpyrene hydroxylase in the duodenum The enzyme activity, however, decreased rapidly in the jejunum (fig 1B) In the small intestinal mucosa of the cat only traces of benzpyrene hydroxylase activity were observed (fig 1C) The guinea pig and mouse livers had the highest benzpyrene hydroxylase activity, while the rat liver showed a 30 per cent lower activity, and the cat and rabbit even less (fig 1D) No marked differences between the liver microsomal proteins among the various species could be detected (table 1)

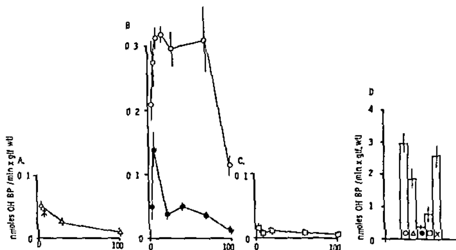


Fig 1 The benzpyrene hydroxylase activity (expressed as nmol of hydroxylated 3 4 benzpyrene (OH BP) $\times$ min.$^{-1}$ $\times$ g (f wt)$^{-1}$) in the small intestinal mucosa of the rat (A) guinea pig (B) mouse (A) rabbit (B) and cat (C) The hepatic benzpyrene hydroxylase activities are given in panel D The numbers of animals were 6 6 8 5 and 4 respectively The distance from the pylorus to the ileocaecal valve has been denoted by 100 The standard errors of the means are shown by the vertical bars The animals are represented by the following signs △=rat ×=mouse ○=guinea pig ●=rabbit and □=cat

Table 1

The hepatic microsomal protein concentrations of different species as expressed in mg of microsomal protein/g (f wt) of liver The standard errors of the means are given The number of experiments is shown in brackets

Species	Hepatic microsomal protein mg/g (f wt)
Cat	25 8 ± 4 9 (4)
Guinea pig	31 0 ± 3 2 (6)
Mouse	35 4 ± 4 0 (7)
Rabbit	32 3 ± 5 5 (3)
Rat	30 0 ± 3 5 (5)

The UDP glucuronyltransferase activity was highest in the small intestine of the rabbit The enzyme activity in this species decreased slowly from the duodenum aborally (fig 2B) The rat showed almost as high an activity but a steeper gradient (fig 2A) The guinea pig and mouse showed equally high mucosal UDP glucuronyltransferase activities, which were only 20 per cent of the enzyme activity found in the rabbit duodenum (fig 2A and B) The small intestinal mucosa of the cat was not able to conjugate *p* nitrophenol (fig 2C) The guinea pig had twice as high hepatic UDP glucuronyltransferase activity as the mouse or rabbit and three times as high as the rat liver (fig 2D) In the cat liver the UDP glucuronyltransferase activity was barely detectable (fig 2D)

Feeding the rats on the rabbit pellets enhanced the intestinal benzpyrene

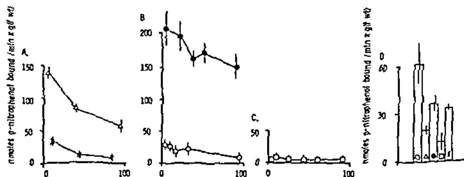


Fig 2 The UDP glucuronyltransferase activity (expressed as nmol of *p* nitrophenol bound as glucuronide × min^{-1} × g (f wt)$^{-1}$) in the small intestinal mucosa of the rat (A) guinea pig (B) mouse (A) rabbit (B) and cat (C) The hepatic UDP glucuronyl transferase activities are shown in panel D For other explanations see fig 1

Table 2

The 3 4 benzpyrene hydroxylase and UDP glucuronyltransferase activities in the duodenal mucosa and liver of rats after feeding the animals on different diets or after a 72 hour starvation period The standard errors of the means are given The number of experiments is shown in brackets The astherisk indicates statistical significance as compared to controls ((x) 0 01 < P < 0 05, (xx) P < 0 01)

Diet	Hydroxylated benzpyrene nmol/min × g (f wt)		p Nitrophenol bound nmol/min × g (f wt)	
	Duodenum	Liver	Duodenum	Liver
Controls	0 065 ± 0 022 (10)	1 99 ± 0 11	170 ± 5 (4)	15 9 ± 1 1
Rabbit pellets	0 340 ± 0 130 (4)(xx)	1.32 ± 0 33(x)	165 ± 13 (5)	16 8 ± 2 6
Dog pellets	0 041 ± 0 010 (4)	1 88 ± 0.25	147 ± 12 (5)	12 8 ± 0 6(xx)
Starvation	<0 010 (4)	1 24 ± 0 10 xx)	108 ± 10 (5)(xx)	12 8 ± 0 6(xx)

hydroxylase to a nearly four fold level as compared with the enzyme activity in rats on an ordinary diet (table 2) On the other hand the dog diet feeding slightly decreased the activity The rabbit diet had no effect on the duodenal UDP glucuronyltransferase activity, and the dog diet decreased it slightly (table 2) The hepatic benzpyrene hydroxylase or UDP glucuronyltransferase activity was not enhanced by the diet of the other species (table 2) Starvation (72 hours) decreased the intestinal benzpyrene hydroxylase activity below the sensitivity of the method, and the duodenal UDP glucuronyltransferase activity decreased some 40 per cent (table 2) Hepatic hydroxylation and glucuronidation also decreased after starving (table 2)

Discussion

The results obtained indicate that there are marked inter species differences in the small intestinal and hepatic drug hydroxylation and glucuronidation The carnivorous cat showed the lowest capacity for drug metabolism and the herbivorous species had quite high ability to metabolize drugs ALVARES *et al* (1970) have previously shown that rabbits have a 50 % lower hepatic benzpyrene hydroxylase activity than guinea pigs They also showed that rats have nearly 3 times and mice nearly twice higher hepatic benzpyrene hydroxylase activities than rabbits These results do not quite agree with our own The discrepancy may be due to the differences in the genetic background and perhaps also to the environments of the tested animals However, the *in vitro* demethylation of ethylmorphine by liver microsomal preparations of mouse, rat, guinea pig, and rabbit showed a

4 fold variation in rate, the preparations from the mouse being most active and least active in the rabbit (DAVIES *et al* 1969) This agrees very well with the hepatic levels of another mixed function oxidase enzyme, benzpyrene hydroxylase, studied in the present report WATTENBERG *et al* (1962) have reported that guinea pigs have a slightly lower duodenal benzpyrene hydroxylase activity than mice, but that rats have about a 5 fold activity as compared with guinea pigs Their results are thus not in accordance with our own

The inter species variations in drug metabolism are most probably due to genetic factors and differences in exposure to inducers Benzpyrene hydro xylase has been shown to be genetically controlled (GIELEN *et al* 1972) Furthermore the diet is known to have a marked effects on the enzymatic activities in the gut (WATTENBERG 1971, HIETANEN *et al* 1972) Thus the difficulty in keeping other factors than inter species differences constant arises, for example between herbivorous, omnivorous and carnivorous animals WATTENBERG (1971) has shown that intestinal benzpyrene hydroxylase can be induced in rats by dietary constituents, e g especially by the vege tables of the Brassicaceae family In the present study it was found that feeding the rat with the rabbit pellets, enhanced the duodenal benzpyrene hydroxylase activity almost 5 fold

The gastro intestinal bacterial flora is also able to metabolize drugs and other chemicals Although several of these organisms are common to many species, they have been found to vary in numbers and location in the intestine of different animals, and they can vary within the same species according to the nature of the diet (SCHELINE 1968) Thus it is possible that inter-species variations in the metabolism of some compounds may depend partly on the gut flora

Since the guinea pigs and rabbits on one hand, and the mice and rats on the other, were fed on the same diets, the differences in drug metabolism between those two groups are at least partially due to the dietary differences This conclusion is supported by the fact that rabbit pellets enhanced the intestinal benzpyrene hydroxylase in rats to the same level as in guinea pigs The variations between the guinea pig and rabbit and between the mouse and rat, are, however, probably true inter species differences The cat's vegetable free diet probably does not contain the same amounts of inducers as the diet of other species, and this may partly explain the low activities of drug metabolizing enzymes

The high benzpyrene hydroxylase activity at the beginning of the jejunum in the guinea pig and rabbit may be due to the liberation of inducing compounds from the feed or from the intestinal microflora An entero hepatic circulation of inducers may also contribute to the high plateau observed in the lower duodenum and middle parts of the small intestine in these species

The results of the present study indicate that although drug hydroxylation and glucuronidation are subsequent events in drug elimination *in vivo*, the activities of these respective enzymes can vary independently of each other Furthermore high intestinal drug metabolizing activity, too, is not necessarily linked to a high hepatic drug metabolism This is in accordance with the results obtained from the studies where the oxidation and conjugation steps have been found to possess different induction patterns (AITIO *et al* 1972)

Acknowledgements

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Effects of Pyrithioxine, EMD 17246 and Diethanolamine-Rutin on Acute Alcoholic Intoxication in Rats

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Abstract The effects of pyrithioxine EMD 17246 (a derivative of pyrithioxine) and a mixture of diethanolamine and rutin on acute alcoholic intoxication were tested in male Wistar rats A standardized tilting plane test and sleeping time measurements were carried out Neither pyrithioxine nor diethanolamine + rutin counteracted the depressant effect of ethanol Administration of a single dose of diethanolamine + rutin *per os* two hours before testing did not markedly affect motor co-ord nation during inebriation On the other hand motor capa bility was slightly better in animals pretreated orally with pyrithioxine (0 1 g/kg/day) for one week than in control animals Following acute oral administration of 0 005 g pyrithioxine/kg body weight there was also slightly improved per formance but larger doses (0 1 and 2 0 g/kg) made this rather worse An injection of pyrithioxine (0 025 g/kg) or EMD 17246 (0 025 g/kg) given intra peritoneally together with ethanol improved motor co-ordination Blood ethanol concentrations were not sign ficantly affected by pre treatments with the drugs The results suggest that pyrithioxine and EMD 17246 slightly protect rats against acute alcoholic intoxication but the effect appears to be dependent on dose level and mode of administration

Key words Ethanol intoxication – pyrithioxine – EMD 17246 – diethanol amine + rutin

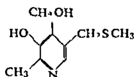
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Many substances including caffeine, amphetamine and pyrithioxine (NOWAK & SCHORRE 1969) have been studied with regard to the protection they afford against ethanol induced intoxication It has been found that the previ ous use of stimulants can to some extent prevent the sedative effect of ethanol but, on the other hand, they cannot improve muscular co-ordination during inebriation (WALLGREN & BARRY 1970) Pyrithioxine, a vitamin B_6 derivative, which is classified as neither a sedative nor a stimulant (SCHREIBER 1965) and which has no pyridoxine like properties (KÖRNER & NOWAK 1967) was recently reported by GOLDSTEIN & DOLCE (1972) to reverse a

number of the effects of ethanol in cats and rabbits including overt coma in the cat Their experiments, in which electrophysiological techniques were used, suggested that the effect of the drug may not be similar in all species owing to different arousal mechanisms in the brain Rabbits had the earliest and most marked changes at the level of the reticular formation, a structure which is generally believed to be involved in the control of muscle tone, while in cats the hippocampus and the amygdala were more affected No performance tests were made and consequently no data are presented on the possible protection afforded by pyrithioxine against ethanol induced disturbances of motor co-ordination

On the other hand, another recent report by GELLER et al (1970) indicates that pre-treatment of rats with a mixture of diethanolamine and rutin protects the animals against the disruptive effect of ethanol on muscle co ordination It was conjectured that the protective action of the drug mixture might be attributable to retardation of the rate of absorption of ethanol from the gastro-intestinal tract but other possibilities, including the effects of acetaldehyde, were considered

These findings seemed to be sufficient to warrant further investigation of the effects of pyrithioxine and diethanolamine + rutin on ethanol induced sedation and disturbances of motor co-ordination In addition EMD 17246, a new derivative of pyrithioxine was kindly provided for us by E Merck Co (Darmstadt, Germany) for evaluation of its capacity to check ethanol-induced intoxication



EMD 17246

S Methyl 5 thiopyridoxol

Materials and Methods

Male Wistar rats of Danish and Finnish origin 4–7 months old which had been given ordinary laboratory food were used for the experiments In different experiments the animals received pyrithioxine (E Merck AG Darmstadt Germany) a metabolite of pyrithioxine (EMD 17246/charge 4 E Merck AG Darmstadt Germany) and a mixture of diethanolamine (Fluka Switzerland) and rutin (E Merck AG Darmstadt Germany) investigating varied doses administered by different routes before the injection of ethanol

The depressant effect of ethanol as evidenced by the sleeping time was tested only in animals which were pre treated with pyrithioxine (0 1 g/kg/day *per os*) for one week or with a single oral dose of diethanolamine + rutin The suspension of d ethanol

amine and rutin was prepared according to GELLER *et al* (1970) by dissolving 5 5 g of diethanolamine and 300 mg of rutin in a small amount of distilled water and adjusting the pH of the solution to 7 3 with concentrated hydrochloric acid When the substances had dissolved, the mixture was diluted with distilled water to a final volume of 100 ml Of this mixture 10 ml/kg body weight was intubated two hours before the start of the test which was begun by injecting 4 g/kg body weight of ethanol (13.3 % w/v in saline) intraperitoneally All the animals fell into apparent coma within three minutes after the injection after which they were placed on their backs on the floor and watched carefully The criteria of arousal chosen were lifting of the neck, turning on to the belly, and getting on to their feet The test was performed in a quiet room

The rate of elimination of ethanol was determined by taking blood samples from the tip of the tail during the sleeping time test 8 hours after the ethanol injection If a rat did not wake up within 8 hours after administration of ethanol, a score of 8 hours was assigned as the time needed to turn on to its belly and to get on to its feet The ethanol concentration was measured by a Perkin Elmer F 40 gas chromatograph

The motor activity of the animals was tested on a tilting plane by a standardized method (ARVOLA *et al* 1958) Ethanol (2 5 g/kg body weight) was given intraperitoneally as a 10 % w/v solution in saline 20 minutes before the first testing on the plane and each rat was tested every 20th minute during 160 minutes The test on the tilting plane was performed each time by the same person who was trained to do it and who did not know which drug had been given to the rats On the preceding day three control tests were performed for each rat, and the performance of the animals during inebriation was calculated as a percentage of the mean of their own control values. Blood samples were taken from the tip of the tail in order to determine the blood ethanol concentration of each rat immediately after the last test on the plane In a series of control studies it was found that chronic oral pre-treatment with pyrithioxine did not affect the rate of absorption of ethanol from the peritoneal cavity Six different series were performed with groups of variously treated rats In each experiment control groups were given only ethanol

The animals in the first series were pre treated with pyrithioxine and diethanolamine + rutin as in those in the sleeping time test In the second series the effect of a large dose of pyrithioxine was tested A single dose of the drug 2 g/kg body weight, was intubated as a 20 % solution in distilled water two hours before testing In the third series a single but much smaller dose of pyrithioxine (0 1 g/kg body weight as a 1 % solution) was given by mouth two hours before testing In the fourth series the dose of pyrithioxine given orally was further reduced to only 0 005 g/kg body weight as a 0 05 % solution administered two hours before testing In the fifth series pyrithioxine was dissolved in ethanol and saline to give a concentration of 0 1 %, and the animals received 0 025 g/kg body weight of the drug together with the ethanol dose 20 minutes before testing The last series was the same as the fifth, with the exception that pyrithioxine was replaced by the same amount (0 025 g/kg) of EMD 17246

Results and Discussion

A standard measure of severely depressant drug effects is the sleeping-time, defined as the duration of time during which the animal fails to show the righting reflex when placed on its back or side This reflex was not

Table 1

Effects of pre treatment with pyrithioxine (0 1 g/kg/day for one week) and diethanol amine + rutin (single dose) on sleep induced by ethanol and the rate of elimination of ethanol Each figure represents the mean of ten rats ± S D

Treatment	Lifting of the neck (min)	Turning on to belly (min)	Getting on to feet (min)	Rate of elimination of ethanol (mg/100 g body wt /hr)
–	41 ± 24	424 ± 68	447 ± 62	21 1 ± 3 8
Pyrithioxine	52 ± 21	414 ± 55	434 ± 57	22 1 ± 4 1
Diethanolamine + rutin	69 ± 40	444 ± 45	451 ± 38	20 7 ± 2 9

considered in the present experiments, since it has been pointed out that after a depressant dose of alcohol the return of the reflex occurs gradually and with relapses (FORNEY *et al* 1962) Table 1 summarizes the duration of sleep of animals pre-treated with pyrithioxine or diethanolamine + rutin Lifting of the neck, turning on to the belly and getting on to the feet were not significantly different between the drug-treated and control groups The results indicate that the coma induced by giving 4 g/kg ethanol intraperitoneally as a single injection cannot be influenced by chronic oral pre treatment with pyrithioxine or by acute oral administration of diethanolamine + rutin Neither do these pre-treatments affect the overall rate of elimination of ethanol in the rats

It has been reported that in rabbits the behavioural sedative effects of ethanol as well as the ethanol-induced changes in EEG can be entirely prevented by pyrithioxine (0 05 g/kg intravenously, GOLDSTEIN & DOLCE 1972) In these animals, the sedative effects of ethanol seem to appear earliest at the level of the reticular formation In the same experiments it was also found that in cats ethanol affected the limbic system more than the reticular formation and that in the latter animals pyrithioxine prevented the effects of ethanol on EEG even when ethanol was given in doses of 3 g/kg These electro-physiological studies also revealed that pyrithioxine, even when administered chronically, does not affect sleep features, i e neither slow wave sleep nor paradoxical sleep, in cats On the other hand, in a previous paper MULLER CALGAN & HOTOVY (1962) reported that pyrithioxine (0 05 g/kg *per os*) had slight sedative effects in cats Our studies with rats indicate that in these animals the ethanol-induced sleeping-time is neither significantly decreased nor increased by pyrithioxine

As can be seen in table 1, relatively low rates of elimination of ethanol were observed in the rats used in this study, as compared with previous

findings in our laboratories and elsewhere (for a review about alcohol elimination in various animal species, see WALLGREN & BARRY 1970). This unexpectedly low rate of elimination of ethanol in the animals was believed to be due to a difference between Wistar rats of Finnish and Danish origin in the rate of ethanol elimination. A control experiment was therefore made in which 1.5 g/kg of ethanol (10 % w/v solution in saline) was injected intraperitoneally into five animals of each stock. The mean values ± S.D. of the rates of elimination of ethanol were found to be 34.2 ± 2.7 mg/100 g body weight/hr in Finnish rats and 26.0 ± 5.0 in Danish ones ($P < 0.05$ for the difference) which confirms that a difference exists between the stocks.

The effect of ethanol on the performance of rats pre-treated with pyrithioxine and diethanolamine + rutin similarly to those in the sleeping time test is presented in fig. 1. During the whole test period, the performance of the pyrithioxine treated rats was better than that of the control animals. A comparison of the average performances by means of Student's t-test showed a significant effect of pyrithioxine ($P < 0.05$). A single oral dose of diethanolamine + rutin did not seem to alter significantly the motor performance of the animals on the tilting plane. The blood ethanol concentrations after the test were found to be 53.0 ± 3.7, 53.8 ± 1.5 and 55.7 ± 2.6 μmol/ml of blood, respectively, in the diethanolamine + rutin,

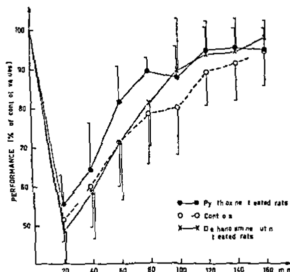


Fig. 1. Effects of chronic pre-treatment with pyrithioxine (0.1 g/kg/day) and an acute single dose of diethanolamine + rutin on performance of rats on the tilting plane after intraperitoneal injection of 2.5 g alcohol/kg body weight. Each point in the figure shows the mean performance of 10 animals. Vertical bars indicate + or − S.D.

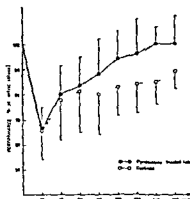


Fig 2 Performance of rats on the tilting plane after intraperitoneal injection of 2.5 g alcohol/kg body weight The pyrithioxine treated rats received 0 005 g/kg of the drug 2 hrs before the first testing. Each point in the figure shows the mean performance of 10 animals. Vertical bars indicate + or − S D

control and pyrithioxine groups (n = 10 in each group) Individual performances of the rats on the tilting plane did not correlate with the measured blood alcohol concentrations, which were determined at the end of the tests It must also be pointed out that the large variation in performance between individual animals was at least partly due to the large qualitative and quantitative variations in behavioural symptoms during inebriation

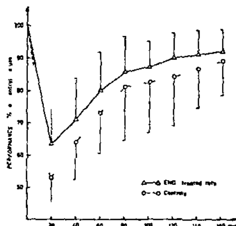


Fig 3 Performance of rats on the tilting plane after intraperitoneal injection of 2.5 g alcohol/kg body weight. Effect of a small intraperitoneal dose of EMD 0 025 g/kg administered together with ethanol Each point in the figure shows the mean performance of 20 animals Vertical bars indicate + or − S D

Contrary to previous findings (GELLER *et al* 1970), we could not protect rats against acute alcohol induced intoxication by pre treating them with a single oral dose of diethanolamine and rutin All our animals showed poor motor co-ordination, ataxia and lethargy soon after the ethanol injection In the experiments made by GELLER *et al* (1970) both ethanol and the drug mixture were administered *per os* and the authors considered it possible that the mixture of diethanolamine and rutin caused a gradual build-up of ethanol in the blood in contrast to the rapid build-up following the administration of ethanol alone Our results support this assumption and suggest that further investigations are needed to establish the mode of action of diethanolamine + rutin on ethanol absorption from the gastro-intestinal tract

A massive dose of pyrithioxine (2 g/kg) given *per os* about two hours before the ethanol injection did not improve the motor function of the rats on the tilting plane Throughout the test the performance of the control rats appeared to be better than that of the pyrithioxine-treated rats At 80, 100 and 120 minutes after the ethanol injection, the difference was even statistically significant ($P < 0 05$), and also the average performance was significantly different ($P < 0 05$) Moreover, it was found in further experiments that even 5 % of the earlier amount of pyrithioxine when given *per os* two hours before the test seemed to worsen rather than improve the animals' performance Up to 60 minutes, the performance of the pyrithioxine treated rats on the tilting plane was nearly as good as that of the control rats, but at 80 and 100 minutes it was significantly worse ($P < 0 05$) Further reduction of the pyrithioxine dose to 5 mg/kg led to a slight improvement in performance, significant at 100, 120, 140 and 160 minutes ($P < 0 05$, $P < 0 05$, $P < 0 001$, $P < 0 01$ respectively) after the administration of ethanol It has been reported that after oral administration pyrithioxine (67–87 mg/kg) is rather rapidly absorbed from the gastro-intestinal tract of Wistar rats and the blood level reaches its peak in the 2nd hour (DARGE *et al* 1969) However, at this time only 0 4 % of the administered dose is found in the blood The substance is relatively rapidly excreted into the urine (75 % within 9 hours) both when administered intravenously or orally (DARGE *et al* 1969) After intravenous administration as much as 95 % of the dose of pyrithioxine is eliminated from the blood after only 5 minutes These data suggest that in our experiments, the rats must have been under the influence of pyrithioxine throughout the test period

A smaller dose of pyrithioxine (0 025 g/kg) given together with ethanol intraperitoneally, somewhat improved the performance of the animals on the tilting plane In this experiment the performance of the pyrithioxine-treated rats was somewhat better than that of control rats up to 140 minutes, but only at 20 minutes was the difference significant ($P < 0 05$) A similar effect was seen when EMD 17246, a derivative of pyrithioxine, was injected

together with ethanol instead of pyrithioxine The rats given EMD 17246 showed a better performance than the controls throughout the test period but the difference was statistically significant only at 20 minutes ($P < 0.05$) Blood ethanol concentrations in the animals at the end of this series were 47.7 ± 3.8 and 50.3 ± 4.4 µmol/ml of blood in EMD 17246 treated and control rats, respectively

The combined results presented in this paper suggest that the motor capability of rats during inebriation may be slightly improved by pyrithioxine or its derivative EMD 17246 However, the effects of the drugs are dependent on the way they are administered as well as on the dose given In rats the sedative effects of ethanol when given in doses which induce coma cannot be counteracted by previous chronic oral treatment with pyrithioxine The present results fail to elucidate the mode of action of pyrithioxine on the brain It has been reported that pyrithioxine is able to normalize glucose metabolism in the brain without affecting cerebral circulation (BECKER & HOYER 1966) In animal experiments it has also been shown that the permeability of the blood brain barrier to electrolytes can be altered by pyrithioxine (QUADBECK *et al* 1962) These latter two findings are interesting, since as early as in 1960 TEIJEIRA & MARTINEZ-LAGE reported that pyrithioxine (0 025 g/kg) when given intramuscularly effectively protected mice against alcohol poisoning and coma induced by alcohol Since pyrithioxine has been reported not to increase the frequency of seizures in epileptic patients (HARTMANN VON MONAKOW 1964), it may be of value in the treatment of patients with acute alcohol poisoning However, this possibility remains to be seen as well as the mechanism of the protection afforded by pyrithioxine against acute alcohol poisoning It would be particularly valuable to find an agent which would antagonize severe alcoholic intoxication, but none of the results obtained in the present investigation are encouraging in this respect

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The Effect of Tyramine on the Transport of 5-Hydroxytryptamine in Blood Platelets

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Key words 5 hydroxytryptamine – uptake – tyramine – human blood platelets

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In order to study the effect of tyramine on the *in vitro* uptake and release of platelets 5-hydroxytryptamine (5-HT) in subjects suffering from migraine, we investigated the effect of tyramine on the uptake and release of platelet 5-HT in normal subjects and rats. This paper reports our results regarding the dose-relationship needed for tyramine to affect the 5-HT transport in normal platelets. As well as the well-known releasing effect of tyramine, an inhibitory effect on the uptake of 5-HT was noted at a concentration, at which no release took place. This inhibitory effect cannot be described as competitive.

Endogenous 5-HT in blood platelets can be released by tyramine *in vitro* (BARTHOLINI *et al.* 1961, MCLEAN *et al.* 1963, DA PRADA *et al.* 1965, BAK & HASSLER 1967, MAY *et al.* 1969). Tyramine seems to interfere with the aggregates, present within the intracellular 5-HT storage granules. These intragranular aggregates are composed of 5-HT (in rabbit platelets also histamine) ATP and bivalent metals (PLETSCHER *et al.* 1967). After incubation of 5 HT containing platelet granules with tyramine, tyramine is mainly concentrated within the 5-HT granules (DA PRADA & PLETSCHER 1969, PLETSCHER *et al.* 1971).

As regards the effect of tyramine on 5-HT uptake this has been studied in human platelets (STACEY 1961). When platelets are incubated for 20 min in the presence of EDTA, 5-HT uptake is inhibited to 50 % by 1.1 × 10^{-4} M tyramine. However, neither the mechanism of the inhibition nor the effect on the 5-HT release has been investigated.

We have investigated the tyramine-induced inhibition of the *in vitro* uptake of 5-HT by human and rat platelets at tyramine concentrations of

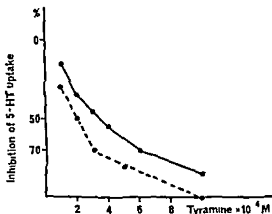


Fig 1 Effect of tyramine on uptake of 5 HT by platelets After pre-incubation for 15 min at 37°, platelets were incubated for 30 min with 1.7×10^{-6} M of 5 HT and different doses of tyramine For human platelets, each point represents the mean of 2–6 experiments Ordinate Percentual inhibition of the 5 HT uptake Abscissa Concentration of tyramine Human platelets ——— Rat platelets ------

$10^{-6}-10^{-4}$ M Furthermore, we investigated the release of 5-HT from human platelets at tyramine concentrations of $2 \times 10^{-4}-10^{-3}$ M Finally the mechanism of inhibition was examined at a tyramine concentration of 2×10^{-4} Triple determinations were carried out in each experiment The 5-HT of the

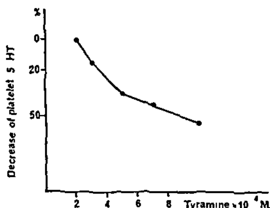


Fig 2 Effect of tyramine on 5 HT release from human platelets After pre incubation for 15 min platelets were incubated for 30 min with different doses of tyramine The 5 HT retained in the platelets after incubation was plotted against the dose of tyramine Each point represents the mean of 2–6 experiments Ordinate Percentual decrease of 5 HT content of platelets Abscissa Concentration of tyramine

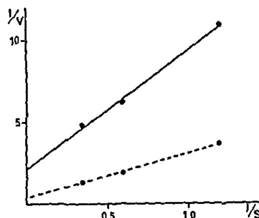


Fig 3 After pre incubation for 15 min, human platelets were incubated for 7 min with 0 85 1 7 and 3 0 μM of 5 HT and 2 × 10⁻⁴ M of tyramine Ordinate The reciprocal of the uptake of 5 HT into platelets during the incubation (l/V) Abscissa The reciprocal of the concentration of 5 HT (l/S) in the incubation medium in the absence of tyramine and in the presence of tyramine ———

platelets was estimated by UDENFRIEND'S method (1955) as modified by CRAWFORD (1963) The fluorescence was measured in an Aminco Bowman *spectrophotofluorometer The platelet counts were performed by means* of phase contrast microscopy (GENEFKE 1971)

No inhibition of the 5-HT uptake was found with $10^{-6}-10^{-5}$ M of tyramine for human or rat platelets Inhibition of human platelets occurred with 10^{-4} M of tyramine With 2×10^{-4} M and 4×10^{-4} M of tyramine, the inhibition was 33 ± 8 % (mean ± S D) and 57 ± 5 %, respectively, and with 10^{-3} M, 85 % Rat platelets seemed to be more sensitive to tyramine with 10^{-4} M and 5×10^{-4} M of tyramine, the inhibition was 30 % and 80 %, respectively (fig 1)

Table 1

Human platelet count before and after incubation with tyramine for a period of 30 minutes

Tyramine (M)	Platelets/ml × 10⁹ Before	After
2×10^{-4}	3 7	3 7
5×10^{-4}	3 8	3 8
7×10^{-4}	3 5	3 4

Table 2

Rat platelet count before and after incubation with tyramine for a period of 30 minutes

Tyramine (M)	Platelets/ml × 10^9 Before	After
3×10^{-4}	6.2	6.1
5×10^{-4}	6.9	6.9

At 2×10^{-4} M of tyramine and below, no release of 5-HT occured for human platelets At 3×10^{-4} M of tyramine, 7×10^{-4} M and 10^{-3} M the release was 15 %, 42 ± 4 % and 55 %, respectively (fig 2)

In an attempt to clarify the mechanism of tyramine inhibition of 5-HT uptake, human platelets were incubated for 7 min with 2×10^{-4} M of tyramine and 3 different concentrations of 5 HT The Lineweaver-Burk plot indicates that the inhibitory effect cannot be described as competitive (fig 3)

The platelet counts were not influenced by incubations with tyramine (table 1 and 2)

In the present investigation, the concentration of tyramine which induced release of 5 HT could be separated from that which inhibited uptake of 5-HT into platelets Whereas 2×10^{-4} M of tyramine had no 5-HT releasing activity, it inhibited the uptake of 5-HT from the medium by 33 % Whether this can be explained by a membrane effect of tyramine as well as to its effect on platelet granules has to be further elucidated

The inhibition of 2×10^{-4} M of tyramine and the release of 7×10^{-4} M of tyramine were selected for further investigations on 5-HT uptake and release in platelets from subjects suffering from migraine (DALSGAARD-NIELSEN & GENEFKE 1972)

Acknowledgements

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Accumulation of Guanethidine by Sympathetic Ganglia of Reserpinized Rats

By

Per Juul

(Received February 26, 1973, Accepted March 10 1973)

Key words Guanethidine – reserpine – sympathetic ganglia

Guanethidine accumulates in the sympathetic ganglia The larger part of the accumulation is sensitive to desmethylimipramine, whereas reserpine only insignificantly lowers the ganglionic content of guanethidine (JUUL & SAND 1973) Since very high doses of reserpine are necessary to deplete the ganglionic stores of noradrenaline (NORBERG 1965) experiments were undertaken to investigate a possible effect of very high doses of reserpine on the accumulation of guanethidine by the sympathetic ganglia

56 male Wistar rats weighing 210–280 g at the initiation of the experiments were used Guanethidine sulphate (ismelin® CIBA, 10 mg/ml in normal saline) was administered intraperitoneally once daily in a dose of 20 mg/kg for 7 days In one experiment the animals received daily intraperitoneal injections of reserpine (serpasil® CIBA) 1 mg/kg one hour before each dose of guanethidine In another experiment a single injection of reserpine 5 mg/kg was administered 4 hours after the last injection of guanethidine The animals were killed 12 hours after the last dose of guanethidine, and the content of guanethidine in the superior cervical ganglia was determined by a fluorimetric method (JUUL & SAND 1973) Each analysis comprised 8 pooled ganglia from 4 rats The results are shown in table 1

It is apparent from the results that reserpine even in high doses neither prevents the ganglionic uptake of guanethidine nor releases the guanethidine already accumulated The lack of effect of reserpine on the storage of guanethidine in the rat sympathetic ganglia seems to indicate that the ganglionic guanethidine is located outside the granular vesicles as opposed to its intragranular storage at the noradrenergic terminals

Table 1

Content of guanethidine base in rat superior cervical ganglia 12 hours after the intra peritoneal adm nistration of guanethidine sulphate 20 mg/kg once daily for 7 days Reserpine was administered as stated Each analysis comprised 8 pooled ganglia from 4 rats Mean values and S E M in brackets n = number of experiments

Drugs doses and time of administration	n	ng guanethidine per ganglion	µg dry weight per ganglion
Guanethidine sulphate 20 mg/kg	5	94 (9 3)	494 (19)
Guanethidine sulphate 20 mg/kg + reserpine 1 mg/kg 1 hour before each injection of guanethidine	4	97 (7 1)	534 (28)
Guanethidine sulphate 20 mg/kg + reserpine 5 mg/kg 4 hours after the last injection of guanethidine	5	113 (8.2)	542 (12)

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The Distribution Pattern of a Series of Tricyclic and Bicyclic Thymoleptics Compared with their Lipophilic Properties and Binding to Plasma Proteins

By

A Jørgensen, V. Hansen and K Fredricson Overø

(Received October 24, 1972, Accepted November 27, 1972)

Abstract Eight thymoleptic drugs were labelled with carbon 14 Four of the drugs were of the tricyclic type (amitriptyline/nortriptyline and melitracen/litracen), the others belonged to the bicyclic phthalane and thiophthalane structures The drugs were given intravenously to male albino rats in doses of 5 mg/kg At fixed times after injection the animals were killed by exsanguination Blood was collected and examined together with different organs for contents of radioactivity Chromatographic estimations were made on extracts of the brain The relative lipophilic properties of the drugs were determined as well as the binding to plasma proteins The concentration of radioactivity in the lungs was in all cases higher after a secondary than after the corresponding tertiary amine In the brain the initial concentrations were highest after the tertiary amines, but at later periods the concentrations were almost the same after a tertiary and the corresponding secondary amine Among the tertiary and the secondary amines, respectively, the drugs of the tricyclic type showed the highest brain concentrations The chromatographic estimations of brain extracts showed that after the administration of a tertiary amine, the parent drug accounted for almost all radioactivity, while after a secondary amine the N demethylation product was present in considerable amounts The lipophility studies showed that the tertiary amines were in all cases much more lipophilic than their corresponding secondary amines The data for the binding to plasma proteins did not show any general trend It is concluded that the penetration of drugs into the brain is only partly explained by the lipophility of the drugs The binding to plasma proteins does not seem to influence the distribution

Particular attention is drawn to the astonishing finding, that more N demethylated metabolites are found after secondary amines than after tertiary amines, though the latter are N demethylated at a higher rate

Key words Thymoleptics – distribution – lipophility – protein binding

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In 1965 a new basic structure was discovered for drugs with thymoleptic properties and in the following years a series of drugs was synthesized and

tested for thymoleptic properties in animals (PETERSEN *et al* 1966 & 1970) Among these drugs the phthalanes and the thiophthalanes turned out to be the most potent The most potent phthalane derivative, Lu 3-010, was superior to the most potent thiophthalane derivative, Lu 5-003, in studies on the peripheral noradrenaline neurones (PETERSEN *et al* 1970, CARLSSON *et al* 1969), while the reverse was the case in the central neurones (CARLSSON *et al* 1969) Because of the higher lipophility of the thiophthalane it was suggested that the concentration of this compound in the brain was higher than the concentration of the phthalane

To investigate this hypothesis the two drugs, both of which are secondary amines, together with the corresponding tertiary amines were labelled with carbon 14 The distribution as well as the lipophility and binding to plasma proteins of the drugs in rats were determined and compared with the data for some of the well-known tricyclic antidepressants

Materials and Methods

The labelled compounds

The structures of the eight compounds investigated are given in fig 1 The labelled compounds were prepared by synthesis from a labelled precursor giving a carbon 14 labelling situated in the methylene group alpha to amine nitrogen as indicated on fig 1 The identity and the radiochemical purity of the labelled compounds were controlled by thin layer chromatography in several systems The data obtained are given in table 1 The compounds were made as water soluble HCl salts and all weights given refer to this salt

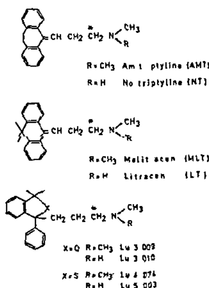


Fig 1 Chemical structures of the drugs and position of labelling (*)

Table 1.

Specifications of the labelled drugs (for formulae and position of labelling see fig 1)

	Specific act (µci/mg)	Radiochemical purity (%)
Amitriptyline (AMT)	6 5	>91
Nortriptyline (NT)	5 7	>96
Melitracen (MLT)	7 9	>95
Litracen (LT)	18 4	>90
Lu 3 009	5 4	>90
Lu 3-010	13 1	>92
Lu 4-074	5 8	>90
Lu 5-003	7 7	>94

Animal experiments

Male albino rats (Wistar/Af/Han/Mol(Han 67)) weighing 80–110 g and fasted for about 16 hours before the administration of the drug, were used in the studies The animals were given a single intravenous injection of the labelled drug, 5 mg/kg body weight in a tail vein Groups of 2 to 3 rats were killed by exsanguination under ether anaesthesia at different times after administration of the drug Blood was collected in tubes containing EDTA to prevent clotting Different organs were removed for examination

Preparation of radioactive samples

The scintillators used were toluene triton X 100 (2 1) containing 6 g/l of PPO and 0 3 g/l of Me_2-POPOP for blood samples and the dioxane-methanol toluene-naphtalene mixture (diotol) described by HERBERG (1960) modified by replacing POPOP with Me_2-POPOP for the tissue samples

Blood To 0 1 ml of blood were added 1 ml of conc NH_3-solution and 0 1 ml of 30 % H_2O_2-solution After gentle shaking the sample was left at room temperature for one hour and then heated for another hour in an oven at 60° After cooling 10 ml of scintillator was added

Tissues Homogenates containing 1 part by weight in 4 volumes of water were made of the brain lungs and other organs A 0 2 ml aliquot was added to 10 ml of scintillator

Extraction and chromatography

Brain homogenates were extracted 3 times with at least twice the volume of dichloro ethane (DCE) after adjusting the pH of the homogenate to 9 or 10 The pooled extracts were concentrated to 3–5 ml at low pressure Aliquots of the concentrated extracts were counted or subjected to thin layer chromatography on Silica Gel G in 3 different solvent systems After development the silica gel was scraped off automatically into separate counting vials for each half centimeter, beginning at the starting point and the radioactivity determined in 10 ml of scintillator

Determination of lipophility

Two different methods for the estimation of lipophility were used

By the first of these the partition of drug between 0 2 M phosphate buffer pH 7 4

and heptane was determined spectrophotometrically at equilibrium after shaking the two phases in a separating funnel

By the other method the partition between water and paraffin was determined by means of a thin layer chromatographic method (MERCIER & DUMONT 1969)

The first method gives an estimate of the lipophility of the drug at pH 7 4, while the latter estimates the lipophility at basic pH i e for the unionized form only

Determination of plasma protein binding

The protein binding of the drugs was determined by ultracentrifugation technique as described by BORGÅ *et al* (1969) Fresh heparinized rat plasma and labelled drugs at a concentration of 1 μg/ml plasma were used Two to six experiments were performed with each drug

Results

As can be seen from fig 1, the studies include 4 pairs (tertiary/secondary amines) of thymoleptic drugs, 2 pairs being of the well-known tricyclic configuration and two others of a new bicyclic type The tricyclic drugs are of the dibenzocycloheptadiene (AMT/NT) and dibenzocyclohexadiene (MLT/LT) type, while the bicyclic drugs belong to the phthalane (Lu 3-009/ Lu 3-010) and thiophthalane (Lu 4-074/Lu 5-003) series

The concentration of radioactivity in the lungs, brain and blood expressed as μg of parent drug/g tissue or ml blood is given in fig 2, a–d In each part of the figure the results for a tertiary amine are depicted together with those for the corresponding secondary amine It is seen that the concentration in the lungs in all cases is higher after the secondary than after the corresponding tertiary amine The same is also true for the concentration

Table 2

Brain/blood ratios for the concentrations of total radioactivity at different times after administration of labelled drug

	5 min	15 min	20 min	60 min	240 min
AMT	6 8		7 1	4 8	2 0
NT	5 7		5 2	4 4	2.2
MLT	10		13	13	5 8
LT	5 0		5 5	6 6	7 4
Lu 3 009		2 8		3 6	0 8
Lu 3-010		0 4		0 4	0.3
Lu 4-074	1.2		1 7	1.5	0 1
Lu 5-003	1.3		2 0	2 6	1 7

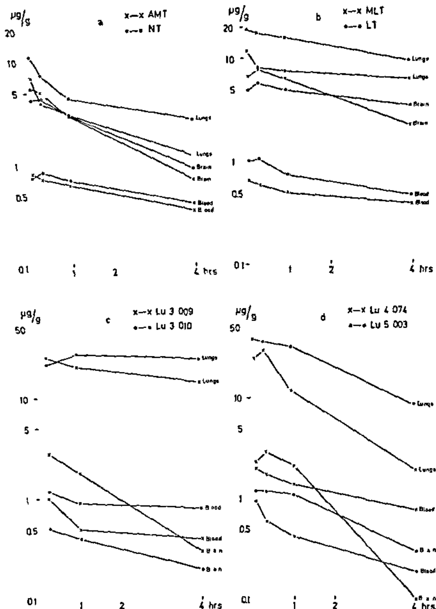


Fig 2 Concentrations of radioactivity expressed as µg of parent drug per g of tissue or ml of blood in lungs, brain and blood for a AMT/NT, b MLT/LT, c Lu 3-009/ Lu 3-010 d Lu 4 074/Lu 5 003 The values are means from groups of 2–3 animals The crosses indicate the groups given a tertiary amine, the open circles groups given a secondary amine

and heptane was determined spectrophotometrically at equilibrium after shaking the two phases in a separating funnel

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LT	5 0		5 5	6 6	7 4
Lu 3 009		2 8		3 6	0 8
Lu 3-010		0 4		0 4	0.3
Lu 4-074	1 2		1 7	1 5	0 1
Lu 5-003	1.3		2 0	2 6	1 7

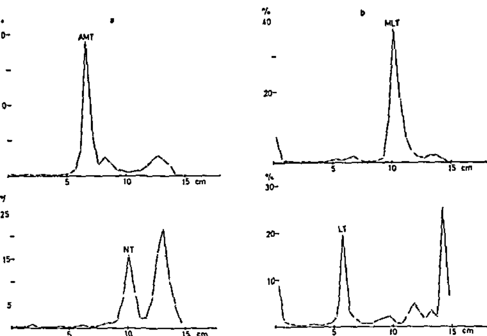


Fig 4 Distribution of radioactivity in the fractions from thin layer plates Abscissa Distance from the starting point Ordinate Radioactivity in each fraction given as per cent of total radioactivity on plate The brain extracts originate from rats given a upper half Amitriptyline, lower half Nortriptyline b, upper half Melitracen, lower half Litracen

Table 3 gives the relative lipophility and degree of protein binding for the eight compounds It is obvious, that the tertiary amines are more lipophilic than the corresponding secondary amines, the factor being 50–100 in the funnel test and 5–15 in the chromatographic test When the pairs are compared, it turns out that the members of the phthalane pair, Lu 3 009/Lu 3 010 are much less lipophilic than the corresponding amines in the other pairs No general trend is seen in the protein binding data, the only astonishing finding being the low binding of LT The reason for this is unknown but a corresponding low binding of this drug is also seen in studies on plasma from the dog and man (FREDRICSON OVERØ, unpublished results)

Discussion

Because of the lipophilic nature of the blood brain-barrier it is obvious that the lipophility of a compound must play a role in its penetration into the

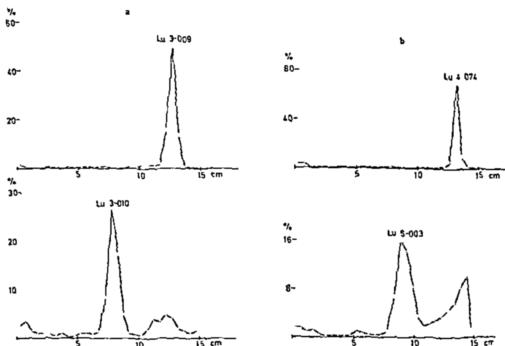


Fig 5 Distribution of radioactivity in the fractions from thin layer plates Abscissa Distance from the starting point Ordinate Radioactivity in each fraction given as per cent of total radioactivity on plate The brain extracts originate from rats given a upper half Lu 3 009, lower half Lu 3-010, b, upper half Lu 4 074, lower half Lu 5 003

Table 3

The relative lipophility of the eight thymoleptic compounds determined as the partition coefficients in heptane/buffer (pH 7 4) and paraffin/water and the plasma protein binding in rat plasma at a drug concentration of 1 μg/ml

	Relative lipophility		Protein binding
	Heptane/buffer 7 4	Paraffin/water	% free drug
AMT	62	5 9	18
NT	1 1	1 0	10
MLT	160	6 8	11
LT	3 4	1 3	43
Lu 3-009	7 1	1 1	29
Lu 3 010	0 079	0 19	23
Lu 4 074	97	15	9
Lu 5-003	1 0	1 0	11

brain The present studies show, in accordance with this view, that the more lipophilic tertiary amines penetrate the blood brain barrier faster than the corresponding secondary amines, giving a higher initial concentration in the brain of tertiary than of secondary amines However, at the later periods after administration, the concentration of the secondary amines is almost the same as the concentration of the tertiary amines, taking into consideration the relatively large amount of metabolites found after injection of the secondary amines A similar change in brain concentrations with time after the administration of a tertiary and the corresponding secondary amine has also been shown for the imipramine/desipramine pair by JORI *et al* (1971) Apparently lipophility only determines the initial rate of the brain uptake of a drug while the concentration obtained after some time is determined by other factors This point of view is in agreement with the statement made by JAHNCHEN & KRIEGLSTEIN (1971) from their studies on perfused, isolated rat brain with promazine, chlorpromazine and their desmethyl products as far as the significance of lipophility is concerned As to the finally obtainable concentration these authors conclude that this value is more dependent on the binding to proteins of the perfusion medium Contrary to this, a comparison of our brain concentrations to the values of the plasma protein binding shows very clearly, that a similar relationship is not observed with our data

When the brain concentrations are compared within the groups of tertiary and the secondary amines respectively, (fig 3), it is evident, that a relationship exists between concentration and lipophility, as far as the tricyclic and the phthalane drugs are concerned, but in this respect the thiophthalane derivatives (Lu 4-074 and Lu 5-003) are exceptions within their groups

Although it is obvious, that the lipophility and maybe also the binding to plasma proteins of a drug play a role in the uptake by the brain, other factors among which should be mentioned pK_A values, biotransformation rates and binding to tissue proteins are presumably also of great importance

The presence of higher amounts of N-demethylated metabolite in the brain after the secondary amines than after the tertiary amines is rather surprising The brain is able to N-demethylate drugs only to an insignificant degree, the published results showing low or no activity depending on the conditions of the studies (FEUER *et al* 1971, CRAMMER & ROLFE 1970, SCHNEIDER & SCHNEIDER 1970, MINDER *et al* 1971) Thus metabolites present in the brain most likely originate from metabolic processes in peripheral organs among which the liver is by far the most important (SCHNEIDER & SCHNEIDER 1970) Since the tertiary amines are metabolized by rat liver microsomes at a much higher rate than the secondary amines (BICKEL *et al* 1967, PLYM FORSHELL *et al* 1968, JORGENSEN & FREDRICSON OVERO, unpublished results) much more metabolite should be available for penetration

into the brain in the case of the administration of tertiary amines A possible explanation to why we, in spite of this, find more N-demethylation products from the secondary amines than from the tertiary amines could be that the half-life for elimination from the brain of a primary amine, formed in the brain or elsewhere, might be much longer than the half-lives for the corresponding tertiary and secondary amines Further distribution studies in which the primary amines are included should be performed to elucidate this question

Acknowledgement

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The Effect of Sodium Chloride on Kidney Function in Rats with Lithium Intoxication

By

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(Received May 12, 1972, Accepted November 13, 1972)

Abstract The effect of lithium was studied in 3 groups of rats given food containing 20 40, or 60 mmol LiCl/kg dry weight for 3–4 weeks The animals had free access to demineralized water The mean serum lithium levels after 3 days were 0 4, 0 7, and 1 1 mM, respectively The rats with low and medium lithium intakes could be maintained at this level for a very long time In the rats with a high lithium intake the serum lithium concentration after 2–3 weeks rose to high values as a result of a decrease in the renal lithium clearance, the rats developed severe lithium intoxication Replacement of demineralized water with 80 mM NaCl solution in severely intoxicated rats reversed the toxic effects the lithium clearance rose, and the serum lithium returned to the same level as observed before the intoxication Administration from the start of the experiment of 20 mM NaCl as the only drinking fluid prevented the development of severe intoxication even in rats given food with 100 mmol LiCl/kg dry weight No decrease in the lithium clearance occurred during an observation period of 8 weeks, and the serum lithium concentration remained constant with a mean value of 1 4 mM The results indicate that the decrease in lithium clearance produced by prolonged administration of high lithium doses is not due to irreversible kidney damage it can be reversed by the administration of sodium The development of severe intoxication can also be prevented by administering sodium along with lithium These observations support the hypothesis that sodium depletion plays an important role in the development of severe lithium intoxication

Key words Lithium – sodium – kidney – intoxication

Lithium is used in long-term maintenance treatment of recurrent manic-depressive disorders (BAASTRUP *et al* 1970, COPPEN *et al* 1971) The drug has a low therapeutic index, and the serum lithium concentration must therefore be held within narrow limits

As lithium is eliminated almost exclusively by renal excretion (RADOMSKI *et al* 1950), the serum lithium concentration is inversely correlated with the renal lithium clearance, it may rise to toxic levels if the renal lithium clear-

ance decreases The lithium clearance is known to decrease in subjects given a low sodium diet and in patients with reduced glomerular filtration rate due to kidney disease (THOMSEN & SCHOU 1968, THOMSEN *et al* 1969) Severe lithium intoxication due to decreased renal elimination has, however, also developed in patients in whom there was no evidence of a preceding low sodium intake or kidney disease The circumstances under which lithium intoxication may develop are thus insufficiently clarified

Studies on dogs and rats (RADOMSKI *et al* 1950, SCHOU 1958a, THOMSEN 1970) have shown that when lithium was given in moderately high doses, the animals developed a pronounced polyuria which remained stable for months At a somewhat higher dose level the animals also developed polyuria but after some time the condition became unstable, the urine flow started to fall and the serum lithium concentration started to rise, indicating that lithium was accumulating in the body This condition is in the following designated 'severe lithium intoxication" because the condition invariably leads to death if no precautions are taken

The mechanism underlying the development of severe intoxication is un known, but sodium appears to be involved RADOMSKI *et al* (1950) observed a striking negative sodium balance during administration of toxic doses of lithium They suggested that loss of sodium and water might have contributed to the fatal outcome RADOMSKI *et al* (1950) and SCHOU (1958a) furthermore found that higher lithium doses could be tolerated by animals receiving sodium chloride simultaneously with lithium than by animals given no sodium

In the present study the renal lithium clearance in rats with a pronounced, stable polyuria and in rats with severe intoxication have been investigated The latter group was examined at a relatively early stage of the intoxication, at a time when the serum lithium concentration had risen only moderately This was done in order to avoid the complex changes in kidney function that result from an advanced intoxication with high serum lithium values The effect of sodium chloride on severe lithium intoxication was studied in two groups of rats In one group, the sodium chloride was administered when the lithium intoxication had already developed In the other group, sodium chloride was given prophylactically in order to examine whether high doses of lithium and high serum lithium concentrations could be tolerated for long time when sodium chloride was given simultaneously with lithium

Methods

Male Wistar rats initially weighing 200 g were used The animals were given lithium mixed with the food and prepared by dissolving 2 0 g NaCl and a 5 g cube of dried meat extract in 600 ml water To this 4 ml of cod liver oil and 10 20 30 or 50 m

Table 1.

Experimental design

Group	n					
		Duration	3–4 weeks			
I	10	Food	20 mmol Li/kg	CD		
		Fluid	water			
		Duration	3–4 weeks			
II	18	Food	40 mmol Li/kg	CD		
		Fluid	water			
		Duration	3–4 weeks			
III	20	Food	60 mmol Li/kg	CD		
		Fluid	water			
		Duration	3–5 weeks		5 days	
IV	10	Food	60 mmol Li/kg	CD	60 mmol Li/kg	CD
		Fluid	water		80 mM NaCl 1 day, 20 mM NaCl 4 days	
		Duration	3 weeks		8 weeks	
V	14	Food	40 mmol Li/kg		100 mmol Li/kg	CD
		Fluid	water		20 mM NaCl	

CD = clearance determinations n = number of rats

of 2000 mM LiCl were added. This solution was mixed with 1 kg dry food prepared as a mixture of 24.5 kg milk powder, 36.8 kg casein, 8.4 kg alfalfa flour, 16.6 kg wheat flour, and 3.7 kg chalk. The food contained 20, 40, 60, or 100 mmol of lithium and 110 mmol of sodium per kg dry weight. It was stored in the cold.

Table 1 presents the experimental design for the five groups of rats. Groups I, II, and III received food *ad libitum* containing 20, 40, and 60 mmol lithium per kg dry weight, respectively. The animals had free access to demineralized water. After 3–4 weeks renal lithium clearance tests were done. In addition the creatinine clearance was determined in group III.

Group IV received food containing 60 mmol/kg LiCl for 3–5 weeks. The rats were followed up individually; when the urine flow started to decrease, indicating the development of severe intoxication, the rat was placed in a separate cage and drinking water was substituted by 80 mM NaCl for one day and 20 mM NaCl for 4 days. The lithium clearance was determined before and after the administration of additional NaCl.

Group V received 40 mmol LiCl/kg dry food for 3 weeks and then 100 mmol LiCl/kg dry food, supplemented with 20 mM NaCl in the drinking fluid for 8 weeks before the clearance test. The treatment with 40 mmol LiCl/kg dry food was given in order to bypass the initial period of lithium treatment during which the water intake changes drastically due to development of polyuria (THOMSEN 1970).

All the blood samples (0.2–0.3 ml) for the determination of the serum lithium concentration were drawn from the end of the cut tail at 9 a.m. under light ether anaesthesia.

For clearance determination the urine was collected quantitatively from each rat during 4 hours, starting 30 minutes after the 9 a m blood drawing. Fluid but no food was available during the clearance periods The urine collection funnels were washed with demineralized water to a final volume of 30 0 ml diluted urine A second blood sample was drawn 30 minutes after the end of the clearance period The mean serum lithium value of the two blood samples was used in the calculation of the lithium clearance, only the 9 a m values are presented in the results The creatinine clearance was calculated on the basis of the creatinine concentration in the last blood sample

The lithium concentrations in the blood and urine were determined by flame photometry (AMDISEN 1967, AMDISEN 1971) Creatinine in the serum and urine was determined by a modified Jaffé reaction 300 μl of plasma or diluted urine was deproteinized by the addition of 600 μl of sodium tungstate (2 5 per cent) and 300 μl of sulphuric acid (330 mM) 700 μl of the supernatant was mixed with 75 μl of saturated oxalic acid and 15 mg of Lloyd s reagent suspended in 175 μl of water The supernatant was discarded and the residue washed with 600 μl of 100 mM HCl The supernatant was again discarded, and the Jaffé reaction was carried out on the sediment by adding 500 μl of alkaline picrate reagent consisting of 5 parts of saturated picric acid, 1 part of 2500 mM sodium hydroxide, and 12 parts of water The extinction was measured at 516 nm and the creatinine concentrations were derived from a standard curve

Results

Fig 1A shows the mean body weights of groups I, II, and III at varying times, and also that of a control group not given lithium Group I gained weight at a somewhat slower rate than the controls, group II showed only a small weight gain, and group III lost weight The loss of weight became more and more pronounced during the treatment period

The water intake as a function of time is shown in fig 1B Groups II and III developed a pronounced polyuria with consequent polydipsia, which in group II continued at a high constant level In group III the polyuria polydipsia reached a maximum after 2 weeks and then started to decrease

After 3 days of lithium treatment, the mean serum lithium concentrations in groups I, II, and III were 0 38, 0 72, and 1 05 mM, respectively (fig 1C), the serum lithium concentration was at this time proportional to the lithium content of the food After 3–4 weeks the serum lithium concentration was still about 0 4 and 0 7 mM in groups I and II, respectively In group III some of the rats showed the same serum lithium concentration as after 3 days, but in the majority of the animals it had increased considerably

The rats with increased serum lithium concentrations had lower urine flows and lower lithium clearances than those with unaltered serum lithium, in the latter the lithium clearances were of the same magnitude as those found in groups I and II Fig 2A and 2B show the relations between serum lithium and urine flow and between serum lithium and lithium clearance

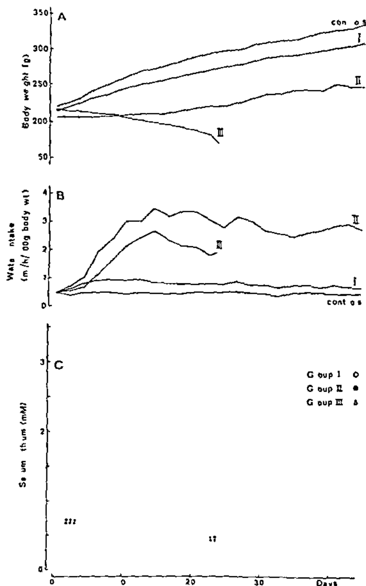


Fig 1A. Average body weight as a function of time in four groups of rats Groups I II and III received food containing 20 40 and 60 mmol lithium per kg dry weight respectively and the controls received the same food without lithium
Fig 1B Mean water intake in the same 4 groups of rats during the experimental period
Fig 1C The individual serum lithium concentrations in the three groups measured at day 3 and after 3–4 weeks

Increased serum lithium, decreased urine flow and decreased lithium clearance are clearly associated phenomena in severe lithium intoxication

The rats with low lithium clearances in group III also had low creatinine

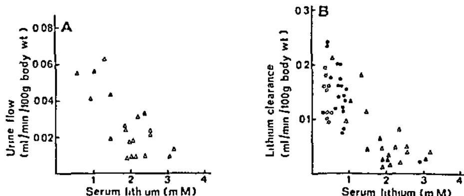


Fig 2A Relation between serum lithium and urine flow in rats from group III given lithium for 3–4 weeks

Fig 2B Relation between serum lithium and lithium clearance in all rats given lithium for 3–4 weeks ○ group I, ● group II, △ group III

clearances (fig 3) The lithium clearances were lowered relatively more than the creatinine clearances and the lithium/creatinine clearance ratio was lowest in the most intoxicated rats

The administration of sodium chloride to rats with severe lithium intoxication (group IV) reversed the changes in serum lithium, lithium clearance, and urine flow Fig 4 (left) shows that the serum lithium concentration decreased to an average level of 1 0 mM, this level is almost the same as the level of 1 1 mM found before the intoxication when lithium had been given

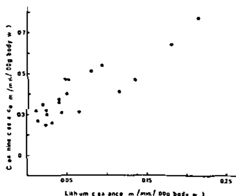


Fig 3 Relation between lithium clearance and creatinine clearance in rats from group III given lithium for 3–4 weeks

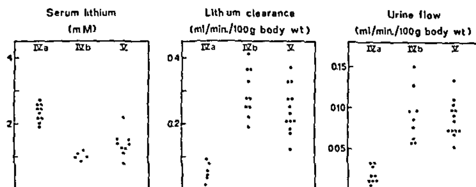


Fig 4 Serum lithium concentration in a blood sample taken before the clearance test, lithium clearance, and urine flow in rats with severe intoxication (group IV) before (a) and after (b) administration of sodium chloride, and in rats given sodium chloride preventively for 8 weeks (group V)

for only one week The decrease in serum lithium was due to a pronounced increase in lithium clearance (fig 4, middle) The urine flow also increased (fig 4, right) The effect of sodium set in rapidly, the mean water intake rose from 1 2 to 10 8 ml/hour/100 g body weight within the first 24 hours of the sodium treatment The ingestion of sodium chloride also reversed the weight loss, the mean body weight rose from 149 g to 181 g in 5 days

Most of the rats in group V, given food containing 100 mmol lithium per kg dry weight and 20 mM NaCl as drinking fluid, did not develop severe intoxication, their serum lithium concentration remained stable at about 1 4 mM (table 2) In a few rats, No 2, No 12, and No 14, the serum lithium concentration at a certain point started to rise They were then given additional sodium chloride, and this stopped the impending intoxication, the serum lithium concentration fell to the previous level and did not rise again (fig 4, right) The effect of sodium set in rapidly, the mean water intake rose lowered, in fact they were increased in comparison with the lithium clearances measured in groups I and II The high lithium clearances were accompanied by high urine flows (fig 4, right) The rats in group V gained weight from a mean value of 254 g to a mean value of 303 g during the 8 weeks they received food containing 100 mmol lithium chloride/kg dry weight

Discussion

The results of the present study show that long-term treatment of rats with lithium chloride leads to changes in kidney function When high doses are given, severe functional alterations with decreasing lithium clearance

Table 2

Individual serum lithium concentrations in rats given food containing 100 mmol of lithium per kg dry weight and drinking fluid containing 20 mmol of sodium chloride per liter (group V)

	Serum lithium concentration (mM)					
Day No	8	12	22	34	46	56
Rat No						
1	1 5	1.5	1 4	1.5	1.5	1 4
2	1 3	1 6	1 4	1 6	1 9	2 2 (a)
3	1 5	1 5	1 4	1 6	1.5	1 6
4	1 1	1.5	1.3	1 0	1 4	1.3
5	1 6	1 6	1 8	1 7	1.5	1.5
6	0 9	1.2	1 1	1 0	1 1	0 8
7	1 0	0 9	1 1	1.2 (b)		
8	1 2	1 6	1 5	1 2	1 4	1 4
9	1 2	1.3	1 7	1.5	1 4	1.5
10	0 9	1.5	1.5	1.4	1 4	1 4
11	1 5	1 4	1 4	1 4	1 6	1 4
12	1.5	2 0	1 7	1 7	2.5 (a)	1 1
13	1 5	1 4	0 8	1 6	1 6	1 6
14	1 6	1 7	2 7 (a)	0 7	1 8	1.3
Mean	1 3	1 5	1 5	1 4	1 6	1 4

(a) In rats No 2 12 and 14 the serum lithium concentration at a certain point began to rise Rats No 12 and 14 were then given additional sodium chloride in the drinking fluid Rat No 12 was given 30 mM NaCl from day 47 to day 56 and rat No 14 was given 80 mM NaCl from day 26 to day 39 and then 30 mM until day 56

(b) Rat No 7 died on day 36 serum lithium was 1 3 mM in a blood sample taken a few minutes after death

occur, and lithium accumulates in the body The animals develop polyuria before they become intoxicated This might give the impression that the polyuria *per se* leads to severe intoxication This is, however, not the case, since dogs and rats can survive with a pronounced lithium induced polyuria for many months without developing intoxication (Radomski *et al* 1950, Thomsen 1970)

The reduction in lithium clearance during severe intoxication was associated with a reduction in the creatinine clearance In previous experiments (Radomski *et al* 1950, Schou 1958a) an accumulation of serum non-protein nitrogen and urea occurred during the late stages of intoxication These observations indicate that the glomerular filtration rate is reduced in severe lithium intoxication

The lithium clearance was lowered relatively more than the creatinine clearance The consequent lowering of the lithium/creatinine clearance ratio shows that during severe lithium intoxication there is an increased fractional reabsorption of lithium in the kidney tubules

The decrease in urine flow observed during severe intoxication is in accordance with the observations of RADOMSKI *et al* (1950) and SCHOU (1958a) The decreases in urine flow and creatinine clearance show that the toxic changes in kidney function involve not only the renal handling of lithium, thus the kidney function appears to be more generally influenced by the ingestion of high amounts of lithium

When sodium chloride was administered to severely intoxicated rats, the lithium clearance and urine flow rose to levels comparable to those found in group V The toxic changes produced by lithium are accordingly reversible

In the experiments carried out by SCHOU (1958a) an irreversible stage of intoxication was reached The most likely explanation for this seeming discrepancy between SCHOU's and the present findings is the different ways of administering lithium SCHOU gave intraperitoneal injections leading to very high serum concentration peaks, and these might have been responsible for the irreversible kidney damage By administering lithium in the food such peaks are avoided, and these circumstances are more comparable to those found in the treatment of human subjects In view of the use of lithium as a drug it is important that the kidney damage should be reversible

Some of the data presented here might indicate that severe lithium intoxication develops when the serum lithium concentration at which the animals are maintained exceeds a particular critical level The rats in group II were maintained at a serum concentration of 0 7 mM and did not develop intoxication the rats in group III were maintained at 1 1 mM and did develop intoxication Hence the critical serum lithium level was under these experimental conditions somewhere between 0 7 and 1 1 mM

However the critical serum lithium level could be raised by altering the experimental conditions, namely by administering additional sodium chloride The rats in group V received extra sodium and tolerated a maintenance serum lithium level of 1 4 mM for more than eight weeks This shows that sodium can protect against severe lithium intoxication in two ways (1) by increasing the lithium clearance and thereby lowering the serum lithium concentration, and (2) by counteracting directly the toxic lithium effect on the tissues so that higher serum lithium concentrations are tolerated

It is not known why the lithium clearance falls during intoxication and why sodium chloride counteracts directly the toxic effects of lithium Lithium clearance is lowered in patients with chronic glomerulonephritis or pyelonephritis Under those circumstances, where the lowering is due to a fall in the number of functioning nephrons, the lithium/creatinine clearance ratio

is either unaltered or increased In severe lithium intoxication, on the other hand, the lithium/creatinine clearance ratio is decreased This indicates that the decrease in lithium clearance during intoxication is not due to a reduction in the number of functioning nephrons

A decrease in the lithium clearance and in the lithium/creatinine clearance ratio is regularly observed in rats with sodium depletion due to the intake of a low sodium diet (SCHOU 1958b) The lithium/creatinine clearance ratio is reduced to a level comparable to that found during severe lithium intoxication It is therefore possible that sodium depletion is responsible for the lowering of the lithium clearance and hence for the development of severe intoxication A striking progressive sodium depletion occurred in the experiments of RADOMSKI *et al* (1950), in which dogs were given toxic doses of lithium

The present experiments involving the administration of sodium chloride were carried out on the assumption that extra sodium chloride might reverse the hypothetical sodium depletion and hence counteract severe intoxication The results show that sodium chloride reverses both the lowered lithium clearance and the lowered urine flow Moreover, administration of sodium simultaneously with lithium prevents severe intoxication even in rats with a higher intake of lithium and a higher serum lithium concentration than those tolerated in the absence of extra sodium chloride This suggests that sodium depletion plays a role in the development of severe lithium intoxication

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Adrenergic and Serotoninergic Mechanisms in Gastric Secretion in Rats

By

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Abstract The influence of adrenergic and serotoninergic mechanisms on gastric secretion was investigated using fistula and pylorus ligated rats Secretion was increased by the tyrosine hydroxylase inhibitor α methyltyrosine This effect could be reversed by L-DOPA 6-hydroxydopamine also increased gastric output The tryptophane hydroxylase inhibitor *p* chlorophenylalanine reduced gastric output and abolished the increase in gastric secretion induced by α methyl tyrosine

The antisecretory effect of serotonin was found to be antagonized by α methyl tyrosine Pre treatment with 6 hydroxydopamine also diminished the anti secretory effect of serotonin

The results indicate the possibility of the existence of an inhibiting peripheral adrenergic tonus in the regulation of gastric secretion in rats Furthermore it is suggested that the anti secretory effect of large doses of serotonin may be due to displacement of noradrenaline in catecholamine neurones Finally the question is raised whether a balance between serotoninergic and adrenergic mechanisms in the regulation of gastric secretion may exist in rats

Key words Gastric acid secretion – rats – catecholamines – serotonin

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The influence of the biogenic amines and the sympathetic nervous system on gastric secretion in rats, has not been subject to the same intensive research as that of parasympathomimetic drugs and the parasympathetic nervous system Generally the catecholamines and serotonin are described as inhibitors of gastric secretion in experimental animals (HÅKANSON *et al* 1967, BASS & PATTERSON 1967, MISCHER *et al* 1969, SHAY *et al* 1958, WINSHIP & SHUCK 1970, FJALLAND 1972) Some investigators have made efforts to elucidate the anti secretory mode of action of the

but it is still questionable whether the catecholamines possess a direct anti-secretory effect (BASS & PATTERSON 1967, PRADHAN & WINGATE 1962) or whether this effect is secondary to the vasoactive properties of the amines (HAIGH & STEEDMAN 1968, JACOBSEN *et al* 1967) However, in a recent paper CURWAIN & HOLTEN (1972) showed, that in dogs the anti-secretory effect of isoprenaline was not due to a decreased mucosal blood flow, whereas the decrease in gastric secretion produced by noradrenaline was probably secondary to the decrease in mucosal blood flow

In most of the experiments published exogenous amines have been used However it is doubtful whether the marked effects of exogenous amines, often administered in rather large doses, have any bearing on their physiological role For this reason the problem has in the present paper been approached by the use of substances which interfere with endogenous amine synthesis, in order to throw some light on the possible function of biogenic amines in the control of gastric secretion in rats

Material and methods

Pylorus ligated rats

Male rats (Wistar/Af/Han/Mol (Han 67) conventional) 150–160 g were fasted for 48 hours in individual cages with free access to water Under ether anaesthesia the pylorus was ligated according to SHAY *et al* (1945) Four hours later the animals were killed under ether anaesthesia, the oesophagus ligated and the stomach removed The gastric juice was collected in centrifuge tubes After centrifugation the volume and the content of total acid were determined A control group receiving saline instead of test substance was included in each experiment

Fistula rats

Male rats (Wistar/Af/Han/Mol (Han 67) conventional) 180–220 g were fasted for 18 hours with water *ad libitum* Under ether anaesthesia a gastric cannula was applied according to the method described by LANE *et al* (1957) The cannula was made of perspex according to a modification of that described by these authors A model constructed by ANTONSEN (personal communication) was used Two weeks after operation the animals were trained for at least four times to remain in a restraining cage Before the experiment the animals were fasted for 18 hours with free access to water They were placed in the restraining cages and the cannulae were provided with a shaft From this the gastric juice was led into a small centrifuge tube placed below the shaft The stomachs were rinsed with 37° warm saline In order to prevent excessive loss of water and chloride during the experiment the animals were given injections of 1 ml saline subcutaneously each hour through a permanently inserted needle After collection of the gastric juice for two hours (basal gastric secretion) the drug under investigation was injected through the needle After the experiment the volume and the content of total acid were determined The control values in the fistula rats varied in the present experiments between 0 7 and 1.2 ml/hr for the volume and between 59 and 98 μeq /hr for the total acid output.

Drugs

The following compounds were investigated and given as aqueous solutions L DOPA methylester hydrochloride 5 HT (serotonin creatinine sulphate) α MT (d 1-α methyl p tyrosine methylester hydrochloride H 44/68) PCPA (d 1 p chlorophenylalanine methyl ester hydrochloride) and 6 hydroxydopamine hydrochloride

Analysis of gastric juice

A volume of 0 5–1 0 ml of gastric juice was used for the determination of content of total acid After titration with 0 01 N NaOH to pH 7 (pH meter Radiometer Copen hagen) the content of total acid was expressed in μeq /4 hours for shay rats and μeq /1 hour for fistual rats

Statistics

The statistical evaluations were done according to Van der Waerdens X test (VAN DER WAERDEN & NIEVERGELT 1956) Determination of ED50 values (experiments with 5 HT) were done by probit analysis as described by FINNEY (1952) The calculations were performed by means of an IBM 1130 computer system using a log dose scale

Results

The influence of the tyrosine hydroxylase inhibitor α MT (SPECTOR 1966) on gastric secretion in fistula rats is shown in fig 1 A marked increase in total acid output was seen in the second, third and fourth hour after 80 mg/kg subcutaneously Administration of L-DOPA (200 mg/kg subcutaneously) resulted in a significant inhibition of output Furthermore it appears that the effect of α MT could be reversed by L-DOPA However a discrepancy exists in the test values for α-MT and α-MT + L-DOPA at the second and third hour This was due to excessive secretion of acid from a single rat in the α MT group But as seen from the figure significant differences first appear at 4 and 5 hours

The effect of repeated dosage with α-MT (50 + 100 + 50 mg/kg intraperitoneally 25, 18 and 2 hours before test) on gastric secretion in shay rats has also been investigated (fig 2) As in fistula rats a significant increase in output was obtained after this compound From fig 2 it can also be seen, that the tryptophane hydroxylase inhibitor PCPA (CORRODI *et al* 1968), given to shay-rats in doses of 100 mg/kg intraperitoneally for three consecutive days preceding the test, significantly decreased the output of volume and total acid. A combination of the two substances resulted in an output not different from the control value

6 Hydroxydopamine, a drug known to destroy temporarily peripheral adrenergic nerve function when given intravenously (THOENEN & TRANZER 1968), increases gastric secretion in pylorus ligated rats (table 1) When given in a dose of 30 mg/kg intravenously

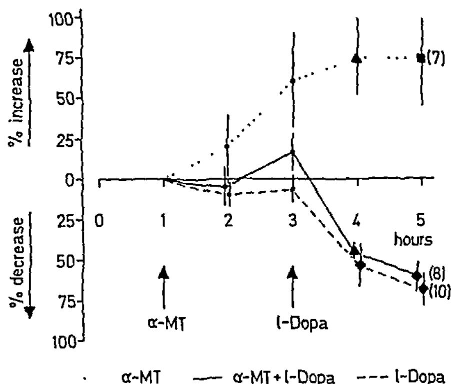


Fig 1 Effect on total acid output in fistula rats of α MT, L-DOPA and a combination of the two substances L-DOPA (200 mg/kg subcutaneously) was given 2 hours after α MT (80 mg/kg subcutaneously) Abscissa time in hours Ordinate per cent change in total acid output. Vertical bars indicate S E M In brackets number of animals used Sign ◆ P < 0 005, ■ P < 0 01, ▲ P < 0 025, ● P > 0 025

Table 1.

Anti secretory effects of 6 hydroxydopamine (30 mg/kg intravenously given 20 hours before test) in pylorus-ligated rats The control group was given saline (1 ml/100 g intravenously) The values are means ± S E M

Treatment	N	Secretory volume ml/4 hr	% increase	Acid secreted μeq /4 hr	% increase
Saline	28	2.6 ± 0 2		236 ± 22	
6-hydroxydopamine	28	3 9 ± 0 3	50* ± 12	368 ± 39	56* ± 17

* P < 0 005

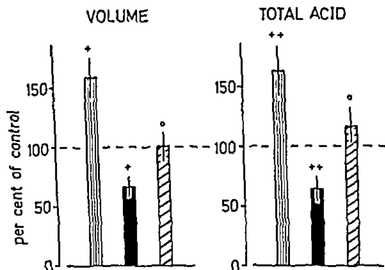


Fig 2. Effect on gastric secretion in pylorus ligated rats expressed as per cent of the control value (broken line – 100 %) α MT (▥) was given 25 18 and 2 hours before test in doses of 50, 100 and 50 mg/kg intraperitoneally (N=22) Control values volume, 2 7±0 2 ml/4 hr, total acid 257±32 µeq /4 hr PCPA (■) was given in three doses of 100 mg/kg intraperitoneally 72, 48 and 24 hours before the test (N=22) Control values volume, 3 1 ± 0.2 ml/4 hr total acid 310 ± 27 µeq /4 hr α MT + PCPA (▨) given in the same doses as mentioned above (N=16) Control values volume, 3 5 ± 0.2 ml/4 hr, total acid 349 ± 36 µeq /4 hr Vertical bars indicate S E M Sign + P < 0 005, ++ P < 0 01, ○ P > 0 025

Table 2

Anti secretory properties of 5 HT in fistula rats

Dose of 5 HT mg/kg s c	N	% inhibition of		ED50 mg/kg s c *	
		Volume	Total acid	Volume	Total acid
5	15	75	83		
2	10	33	47		
0 1	8	39	48	1 33	0 63
0 01	6	8	0	(0 74–2 39)	(0 42–0 95)
0 001	6	2	0		

* In brackets 95 % confidence limits

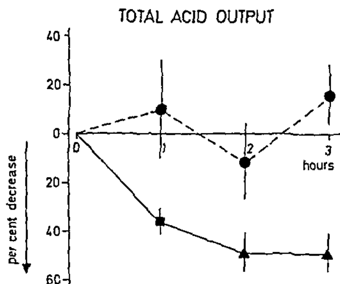


Fig 3 Effect of α MT on the anti secretory effect of 5 HT in fistula rats ——— 5 HT (2 mg/kg subcutaneously) (N = 10), - - - - - - 5 HT (2 mg/kg subcutaneously) + α MT (50 + 100 + 50 mg/kg intraperitoneally 25, 18 and 2 hours before the test) (N = 8) Abscissa time in hours after administration of 5 HT Ordinate per cent change in total acid output Vertical bars indicate S E M Sign ▲ $P < 0\,005$, ■ $P < 0\,01$, ● $P > 0\,025$

6-hydroxydopamine caused a significant increase in the volume and total acid output

5-HT inhibits gastric secretion in fistula rats (table 2), although the ED50 values are quite high When treating fistula rats with α-MT (50 + 100 + 50 mg/kg intraperitoneally 25, 18 and 2 hours before the test), the anti-secretory effect of 5-HT (2 mg/kg subcutaneously) could be completely antagonized (fig 3) It can be seen, that 5-HT significantly reduced the output of total acid, whereas in α MT pretreated rats 5-HT failed to reduce acid output The effect of 5-HT on the volume was similar to the effect on total acid, but according to table 2 less pronounced The inhibitory effect of 5 HT on volume was also antagonized by α-MT pretreatment

The influence of 6-hydroxydopamine on the anti-secretory effect of 5-HT was studied in fistula rats (fig 4) 5-HT (2 mg/kg subcutaneously) was examined for its effect on total acid output before and 1, 8, 14, 21 and 28 days after an intravenous injection of 30 mg/kg of 6-hydroxydopamine It was found, that 6-hydroxydopamine reduced the anti-secretory effect of 5-HT One, 8, 14 and 21 days after the administration of 6-hydroxydopamine the total acid output under the influence of 5-HT was not significantly different from that of controls In animals not treated with 6-hydroxydop-

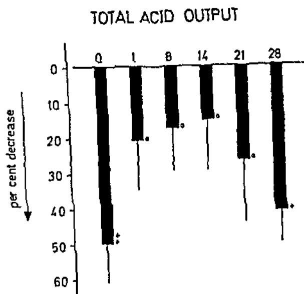


Fig 4 Effect of 6 hydroxydopamine (30 mg/kg intravenously) on the anti secretory effect of 5 HT (2 mg/kg subcutaneously) in fistula rats The effect of 5 HT was estimated before (0) and 1, 8, 14 21 and 28 days after the administration of 6 hydroxydopamine The same animals were used in all experiments (N = 8) The columns represent the per cent inhibition in total acid output by 5 HT Vertical bars indicate S E M Sign ++ P < 0 005, + P < 0 01, ○ P > 0 025

amine and those pretreated 28 days previously, 5-HT caused a significant reduction in acid secretion

Discussion

In the present paper the tyrosine hydroxylase inhibitor α-MT increased gastric output in fistula- and pylorus-ligated rats Moreover it was shown, that the increase in output induced by α MT could be reversed by L-DOPA in fistula rats In shay-rats L-DOPA also antagonized the effect of α-MT (Fjalland & Pedersen 1971) α-MT selectively reduced the central and peripheral noradrenaline level without lowering the 5-HT level (Spector *et al* 1965a, Spector 1966, Herman 1970) This may indicate, that the increase in gastric secretion produced by α MT is due to an inhibition of the synthesis of the catecholamines, and hence due to a reduction in adrenergic tonus either centrally or peripherally

6 hydroxydopamine, when injected intravenously in doses of 20–30 mg/kg, lowered the peripheral noradrenaline content by about 90 % (Thoenen &

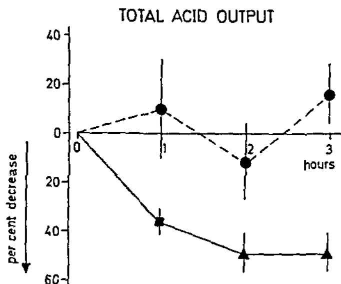


Fig 3 Effect of α MT on the anti secretory effect of 5 HT in fistula rats ——— 5 HT (2 mg/kg subcutaneously) (N = 10), - - 5 HT (2 mg/kg subcutaneously) + α MT (50 + 100 + 50 mg/kg intraperitoneally 25, 18 and 2 hours before the test) (N = 8) Abscissa time in hours after administration of 5 HT Ordinate per cent change in total acid output Vertical bars indicate S E M Sign ▲ $P < 0.005$, ■ $P < 0.01$, ● $P > 0.025$

6-hydroxydopamine caused a significant increase in the volume and total acid output

5-HT inhibits gastric secretion in fistula rats (table 2), although the ED50 values are quite high When treating fistula rats with α-MT (50 + 100 + 50 mg/kg intraperitoneally 25, 18 and 2 hours before the test), the anti-secretory effect of 5-HT (2 mg/kg subcutaneously) could be completely antagonized (fig 3) It can be seen, that 5-HT significantly reduced the output of total acid, whereas in α-MT pretreated rats 5-HT failed to reduce acid output The effect of 5-HT on the volume was similar to the effect on total acid, but according to table 2 less pronounced The inhibitory effect of 5-HT on volume was also antagonized by α-MT pretreatment

The influence of 6-hydroxydopamine on the anti-secretory effect of 5-HT was studied in fistula rats (fig 4) 5-HT (2 mg/kg subcutaneously) was examined for its effect on total acid output before and 1, 8, 14, 21 and 28 days after an intravenous injection of 30 mg/kg of 6-hydroxydopamine It was found, that 6 hydroxydopamine reduced the anti-secretory effect of 5-HT. One, 8, 14 and 21 days after the administration of 6-hydroxydopamine the total acid output under the influence of 5-HT was not significantly different from that of controls In animals not treated with 6-hydroxydop-

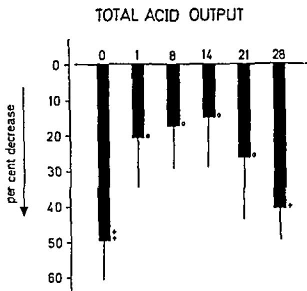


Fig 4 Effect of 6 hydroxydopamine (30 mg/kg intravenously) on the anti secretory effect of 5 HT (2 mg/kg subcutaneously) in fistula rats The effect of 5 HT was estimated before (0) and 1, 8, 14, 21 and 28 days after the administration of 6 hydroxy dopamine The same animals were used in all experiments (N = 8) The columns re present the per cent inhibition in total acid output by 5 HT Vertical bars indicate S E M Sign ++ $P < 0\,005$, + $P < 0\,01$, ○ $P > 0\,025$

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Discussion

In the present paper the tyrosine hydroxylase inhibitor α-MT increased gastric output in fistula- and pylorus-ligated rats Moreover it was shown, that the increase in output induced by α-MT could be reversed by L DOPA *in fistula rats In shay-rats L-DOPA also antagonized the effect of α MT* (FJALLAND & PEDERSEN 1971) α-MT selectively reduced the central and peripheral noradrenaline level without lowering the 5 HT level (SPECTOR *et al* 1965a, SPECTOR 1966, HERMAN 1970) This may indicate, that the increase in gastric secretion produced by α MT is due to an inhibition of the synthesis of the catecholamines, and hence due to a reduction in adrenergic tonus either centrally or peripherally

6-hydroxydopamine, when injected intravenously in doses of 20–30 mg/kg, lowered the peripheral noradrenaline content by about 90 % (THOENEN,

TRANZER 1968, CORRODI *et al* 1971) without interfering with 5-HT (VOTAVOVA *et al* 1971) In this situation gastric secretion was increased by about 55 %

Based on these results the possibility of the existence of an inhibitory peripheral adrenergic tonus in the regulation of gastric secretion in rats is suggested Whether a central component is also involved can not be excluded since α-MT, as mentioned above, exerts its activities both peripherally and centrally

PCPA, the inhibitor of the synthesis of 5-HT, caused inhibition of gastric secretion in shay-rats, an inhibition which has been shown to be antagonized by 5-hydroxytryptophane (FJALLAND & Pedersen 1971) PCPA given in doses of 100 mg/kg for 3–4 consecutive days decreased the central and peripheral 5-HT content by about 80 % without interfering with the noradrenaline content (BRODY 1970, KOE & WEISSMAN 1966) This would indicate, that the inhibition of gastric output by PCPA may be due to a deficiency in the synthesis of 5-HT This does not agree with the above mentioned finding, that 5-HT, itself, decreases gastric secretion However, the doses of 5-HT necessary to inhibit the output were quite large The possibility therefore exists, that exogenous 5-HT in large doses does not inhibit gastric secretion by a direct action

In this study it was shown, that in fistula rats the anti secretory action of 5-HT could be antagonized by pretreatment with α-MT, indicating that in the presence of diminished concentrations of the catecholamines, 5-HT looses it anti-secretory properties Furthermore it was shown, that 6 hydroxydopamine inhibited the anti secretory effect of 5-HT CORRODI *et al* (1971) have shown, that the content of noradrenaline in rat hearts is reduced to 10 % of its initial value 24 hours after 6-hydroxydopamine (20 mg/kg intravenously) After 21 days the content of noradrenaline is 60 % and 28 days after the administration of 6-hydroxydopamine it is 90 % of the original level These results give further supports to the above mentioned assumption, that the anti secretory effect of 5-HT is dependent on the noradrenaline concentration in peripheral adrenergic neurones Based on these results it is proposed, that the effect of exogenous 5-HT in high doses does not reflect the physiological role of 5-HT, but is due to displacement of noradrenaline in the adrenergic neurones

The increase in gastric secretion produced by α-MT has been shown to be antagonized by treatment with PCPA This means, that the two opposite actions of these enzyme inhibitors are abolished by simultaneous treatment It is thus possible that a balance between adrenergic and serotoninergic mechanisms is involved in the regulation of gastric secretion in rats Whether such a regulatory balance is functional in the central nervous system or peripherally cannot be decided on the present data

However, HÅKANSON & OWMAN (1966) have reported, that the 5-HT containing cells in the stomach are not localized closely to the parietal cells, a fact which does not support the hypothesis that peripheral 5-HT may have a regulating role in gastric secretion in rats To clarify these problems further studies are necessary

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Acta pharmacol et toxicol 1973 33, 113–122

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The Ultrastructure of Livers from Chickens Embryogenically Injected with DDT

By

Ove Svendsen

(Received March 1 1973, Accepted March 14 1973)

Abstract DDT (1 1,1 trichloro-2 2 bis(*p* chlorophenyl)ethane) embryogenically injected into eggs causes hepatic fine structural changes in one to three days old chickens as compared with appropriate controls The most striking changes are proliferation of agranular endoplasmic reticulum with concomitant vesiculation of rough endoplasmic reticulum and prolongation and accentuation of the presence of mitochondrial myeloid bodies The proliferation of agranular endoplasmic reticulum supports the finding that DDT increases the activity of drug metabolizing enzymes in hepatocytes from similarly treated chickens Mitochondrial myeloid bodies in embryonic chicken hepatocytes reported in the literature are only visible after double fixation in glutaraldehyde and osmium tetroxide and disappear after the 17th day of embryonic development In this study they were found in one to three days old chickens but were larger and more numerous in the treated chickens suggesting that the normal occurrence of myeloid bodies in embryonic chicken livers is morphologically an expression of cellular immaturity, which can be prolonged or reinduced by DDT

Key words DDT chicken hepatocytes – mitochondrial myeloid bodies – agranular endoplasmic reticulum

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DDT (1,1,1-trichloro-2,2-bis(*p* chlorophenyl)ethane) and phenobarbital (phenemalum NFN) belong to the most potent inducers of drug metabolizing enzyme activity They are classified as non specific inducers (GILLETTE 1963) and produce ultrastructural changes in hepatocytes after dietary intake in sufficient quantity over a period of time (HERDSON *et al* 1964, BURGER & HERDSON 1966, ORTEGA 1966, KIMBROUGH *et al* 1971) The induction of drug metabolizing enzyme activity caused by DDT and phenobarbital is followed by proliferation of the agranular endoplasmic reticulum in hepatocytes (REMMER & MERKER 1963a, 1963b, 1965, HART & FOUTS 1963, 1965,

Shay, H , D C H Sun & M Gruenstein Effect of serotonin and its precursor on inter digestive gastric secretion in the rat *Fed Proc* 1958, **17**, 146 (580)

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Results

Clinical and necropsy findings

The hatchability was 93 % in group one, 86 % in group two, 71 % in group three and 93 % in group four. The chickens in group three and four showed characteristic DDT-induced tremors on the second and third day after hatching, and were most severe in group four on the third day.

The decrease in relative liver weight during the examination period was the same in all four groups.

Chemical analysis

The content of p.p'.DDT in the livers from group three and four increased very fast during the three days after hatching. In group three the content increased from 403 to 521 p.p.m. DDT (all weights are wet weights) and in group four from 496 to 1330 p.p.m. DDT. On the first day the yolk sac contained 443 and 2260 p.p.m. DDT in group three and four, respectively and on the third day 1310 and 1740 p.p.m. DDT. The liver and the yolk sac from the analysed chicken in group two contained 0.026 p.p.m. and 0.024 p.p.m. DDT, respectively.

Light microscopy

All the livers were of normal appearance with a marked diffuse fatty infiltration with many small droplets of fat located in all parenchymal cells. The amount decreased during the three days of examination.

Electron microscopy

In groups three and four, large myeloid bodies in the hepatocytes were common. They varied in size, reaching a maximum diameter of 0.6–0.8 μ and were round or elliptical in shape. They consisted of concentrically arranged lamellae of electron dense material (fig. 1, 2 and 3) and were associated with the mitochondria, being located either inside or partly outside. The superficial lamella was most often continuous with the outer mitochondrial membrane. Such mitochondria showed scanty cristae, and sometimes an irregular outline. Only a few myeloid bodies appeared unrelated to the mitochondria, probably due to tangential sectioning. The frequency as well as the size of the myeloid bodies increased during the three days of examination being most conspicuous in group four.

Myeloid bodies with similar configuration and location were found in hepatocytes from all chickens in groups one and two, but they were smaller and contained only a few lamellae (fig. 4). Compared to groups three and four their frequency was low and they were difficult to demonstrate in all

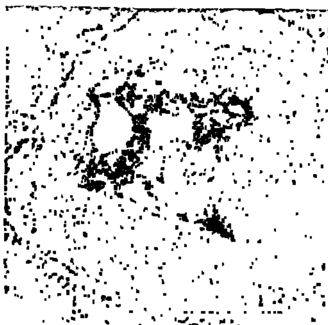


Fig 1



Fig 2.

sections During the three days of examination both frequency and size decreased and on the third day, the myeloid bodies were rarely found

In groups three and four many hepatocytes, especially on the second and third day in group four, contained large vesicles of agranular endoplasmic reticulum (figs 5 and 6) Such vesicles could not be found in groups one and two. In the area of glycogen deposition small vesicles of agranular endoplasmic reticulum were found in all four groups.

In the hepatocytes from groups one and two the rough endoplasmic reticulum consisted largely of single cisternae surrounding almost every mito-

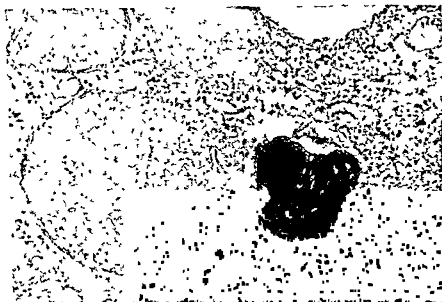


Fig 1–3 Mitochondrial myeloid bodies from the treated groups In fig 1 a myeloid body with very irregular lamellae is situated inside the mitochondria, probably due to section direction The myeloid body in fig 2 is partly surrounded by the inner and outer membranes of the mitochondria At A the myeloid body is in direct contact with the cytoplasm In fig 3 the myeloid body is almost without contact with the membranes of the mitochondria Fig 1 and 3, magnification × 64 000 Fig 2, magnification × 42 000

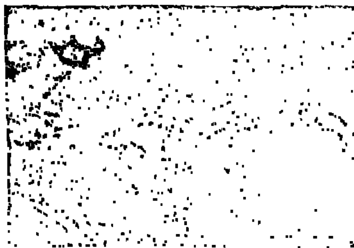


Fig 4 Mitochondrial myeloid bodies (P) from one of the chickens from the control groups Compared to fig 1–3 these myeloid bodies are small and contain only a few, thin lamellae Magnification × 40 000

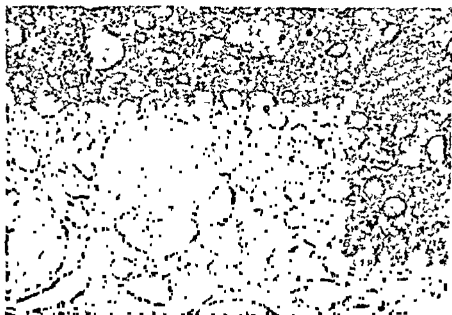


Fig 5-6 Proliferation of the agranular endoplasmic reticulum Group four Some of the free ribosomes are situated near the agranular vesicles (B) Only a few ribosomes are attached to the vesicles (A) In fig 6 the rough endoplasmic reticulum contains electron dense material (C) Magnification × 42 000

chondrion, and of a few vesicles randomly distributed throughout the cytoplasm. The rough endoplasmic reticulum in groups three and four consisted mainly of vesicles located diffusely in the cytoplasm. Some of the vesicles had only a few ribosomes attached and in the cytoplasm more free ribosomes were found as compared to groups one and two. The free ribosomes were often situated near the vesicles and aggregated to form polysomes.

The vesiculated rough endoplasmic reticulum in hepatocytes from groups three and four occasionally contained higly electron dense material, probably protein. Hepatocytes from the same groups had large Golgi complexes consisting of many dictyosomes.

Discussion

The ultrastructure of hepatocytes from groups one and two is, except for the presence of small myeloid bodies, in agreement with that described by STEPHENS & BILS (1967) using osmium tetroxide as fixative. Myeloid bodies like those found in this study are common in embryonic chicken hepatocytes (CURGY 1968) and neuroblasts (CANDIOLLO & FILOGAMO 1966) double fixed in glutaraldehyde and osmium tetroxide. In embryonic chicken hepatocytes fixed only in glutaraldehyde the myeloid bodies are only visible as electron transparent regions, and cannot be found after single fixation in osmium tetroxide (CURGY 1968). After the 17th day of embryonic development myeloid bodies in chicken hepatocytes are rare or absent (KARRER 1961, CURGY 1968). In the present study small myeloid bodies could be demonstrated in hepatocytes from all chickens in groups one and two but on the third day there is a decreased frequency and size as compared with the first day. In hepatocytes from groups three and four the myeloid bodies were more common on the first day and much larger as compared with groups one and two, and became larger and more numerous during the examined period. This must be ascribed to an effect of the injected DDT. Furthermore, it suggests that the presence of myeloid bodies in embryonic chicken cells is a morphological expression of cellular immaturity made visible by fixation in glutaraldehyde and that the cellular immaturity can be prolongated or re-induced by DDT. In rat hepatocytes drug-induced myeloid bodies with different contours have often been described, lately by HRUBAN *et al.* (1972) using osmium tetroxide as fixative. Myeloid bodies like those described in this study have only been demonstrated in cells from embryonic chickens fixed in glutaraldehyde (KARRER 1961, CANDIOLLO & FILOGAMO 1966, WESTMAN & SANDSTRÖM 1966, CURGY 1968).

The presence of vesicles of the agranular endoplasmic reticulum in groups three and four is very common but they could not be found in hepatocytes

from groups one and two except for the area of glycogen deposition The rough endoplasmic reticulum consisted mainly of vesicles, some being nearly devoid of ribosomes The total amount of endoplasmic reticulum membranes was much larger in the treated groups than in groups one and two This may indicate that the increased agranular endoplasmic reticulum in the treated chickens can be transferred from rough endoplasmic reticulum (DALLNER *et al* 1966) The number of free ribosomes in the cytoplasm was increased and many were situated near smooth portions of the rough endoplasmic reticulum This indicates furthermore that some of the smooth vesicles may originate from degranulated rough endoplasmic reticulum This can represent normal proliferative changes of the agranular endoplasmic reticulum (DALLNER *et al* 1966) or desorganization of the rough endoplasmic reticulum (SMUCKLER & ARCASOY 1969) The increase in agranular endoplasmic reticulum is in agreement with the findings of ABOU DONIA & MENZEL (1968a & b), who demonstrated increasing drug metabolizing enzyme activity in liver cell microsomes from chickens embryogenically injected with DDT

The contents of DDT in the liver increased very rapidly from the first to the third day The amount of myeloid bodies and agranular endoplasmic reticulum similarly increased STEPHENS & BILS (1967) have shown a definite increase in the number of lipid droplets present within the hepatocytes after 15 days of incubation These results suggest that absorption of DDT from the yolk sac is slow during the early embryonic period and very fast before and after hatching In embryonic rabbits (HART *et al* 1962) and rats (FEUER & LISCIO 1970) induction of drug metabolizing enzyme activity has only been seen when the mothers are medicated during the final four days of pregnancy CONNEY (1967) suggests a defective enzyme forming mechanism before this time This, too, could explain the late increase of agranular endoplasmic reticulum in the present study

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Perfusion Technique of the Intact Human Rectum*

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Abstract An *in situ* technique for rectal perfusion in humans has been developed The solution is instilled through an outer tube, rises 7.5 cm in the rectum and is drained through an inner tube The method allows well defined absorption conditions with normal physiological function Absorption from two different solutions can be compared in one experiment An example of determining the absorption rate from the appearance of the substance in the plasma is described

Key words Rectal perfusion – *in vivo* technique – man

Perfusion experiments on the gastrointestinal tract in animals have given many data about the absorption and exchange of electrolytes

Because of the greater difficulties and methodical problems such investigations in man are rather scanty However it is highly desirable that the quality and the quantity of the findings are confirmed in humans

As the rectal mucosa is the nearest part of the intestine for direct admittance, and since it is an absorption surface for suppositories, it seemed of interest to find a satisfactory method for rectal perfusion in man

Primarily it is important that the method allows normal physiological functions and ensures well defined absorption conditions Secondly the discomfort to the experimental subject should be negligible and the experiment reasonably easy to perform

Existing methods have been considered, but none of them fulfilled the above mentioned conditions

A common method for examining rectal absorption in humans is to instil

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a test solution in the rectum and either empty the rectum after a certain time or withdraw samples at various intervals Exact results by this method are very difficult or even impossible to obtain, because the absorption surface is poorly defined, and the composition of the solution is changed during the experiment resulting in altered absorption conditions

A double luminal tube with a distendable balloon at the end can be placed in the rectum through the anal canal (DALMARK 1970) Though this method is not directly used for investigation of rectal absorption in the article referred to, it is probably useful for the purpose Yet a well occluding distended balloon may result in an unpleasant defaecation reflex, while the absorption may be decreased by a decreased blood flow due to compression of the rectal wall

Finally, a test solution can be instilled directly into the colon by oral intubation (LEVITAN *et al* 1962) or in colostomy patients (CURRY *et al* 1935) the effusate then being collected through a tube placed in the anal canal It may be possible to perfuse the rectum in the same way, but in addition to a great many practical problems, difficulties in controlling the experimental conditions and unphysiological factors may be involved

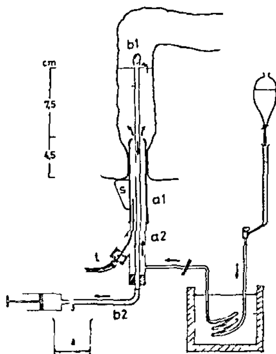


Fig 1 System for rectal perfusion a1 and a2 outer tube b1 inner tube b2 effusate tube s stop block t thermo-feeler



Fig 2 The apparatus used

Materials and methods

Perfusion technique

Description As a result of preliminary experiments the following technique has been chosen A solution is instilled into the rectum by a tube and collected by another tube placed further up in the rectum of the seated experimental subject In this way a well defined area of the rectum is exposed to the solution

Fig 1 is an illustration and fig 2 a photo of the apparatus used An outer tube is placed into the anal canal The infusate is instilled through this tube, and the solution rises up to 7.5 cm in the rectum, where it is drained through tiny holes in an inner tube From a burette the solution is led through a heated water bath to the outer tube

The outer tube consists of two parts (a1 and a2) a1 is made of a reversed poly ethylene test tube (outer diameter 13 mm, length 8 cm) with a hole (diameter 6 mm) at the bottom or of a stiff pointed plastic tube (outer diameter 8 mm, length 10 cm) It should be placed in the anal canal and a plexiglass stop block(s) ensures that the solution is constantly instilled at 4 5 cm from the outer opening of the anal canal a2 is a branched P V C tube (outer diameter 11 mm, inner diameter 8 mm) which holds the thermo-feeler (t), the inner tube and the influx a1, a2, s and branch are glued together with Araldite.

The inner polyethylene tube (b1) (outer diameter 3 5 mm, inner diameted 2 5 mm) is 25 cm long The end of the tube is closed and melted round and smooth in order not to damage the mucosa In the wall of the tube, just beneath the closed end eight small

holes have been made with a needle The diameter of the holes is about 0 5 mm to keep out any rough particles

A 60 cm long effusate tube (b2) (same type as b1) connected with the inner tube leads the effusate into a receiver

Instruction The inside of the inner tube is moistened and the outer tube is filled with the solution from the burette The experimental subject himself inserts the apparatus During the insertion it is an advantage to steady the inner tube with another stiff but thinner tube When the stop block touches the opening of the anal canal the apparatus is fixed to the experimental subject by means of a belt and strings fastened to the outer tube under the stop block The experimental subject is placed on a chair with a circular opening for the tubes surrounded by a ring of foam rubber The effusate tube and the syringe both filled with the solution are now connected to the inner tube and the small draining holes are cleaned by blowing with the syringe The perfusion is then started by opening the tap of the burette and removing the syringe from the effusate tube Drainage which is the main problem, is primarily dependent on the diameter of the draining holes Impurities may block the holes making it necessary to clean them by small blows with the syringe It is better to blow than to suck with the syringe, as sucking usually stops the holes still more Often it may be sufficient merely to shake the effusate tube When the effusate drops regularly the level of the liquid in the rectum is generally correct If small blowing with the syringe as a control results in an abundant flow of effusate the level of the liquid is too high One to two hours after defaecation is the best time for perfusion as at that time the effusate is generally sufficiently clean A perfusion rate of five to six ml per minute is easy to administer but a slow perfusion rate may be preferable in re-circulation experiments with small quantities

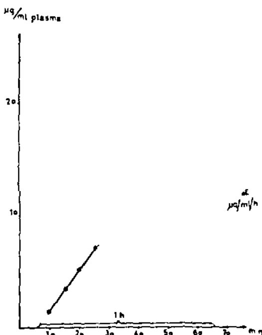


Fig 3 Absorption from a 47 mM salicylate solution (pH 5 9) in one experiment

Determination of absorption

The absorption can be expressed in two ways either as the appearance of the substance in the blood or as the disappearance of the substance from a solution re circulated in the rectum

Fig 3 is an example of the first type of experiment The concentration in the rectum of a salicylate solution is kept constant by perfusion at a rate of 5–6 ml per minute The absorption is determined by examining the plasma salicylate concentration

The total perfusion time is 25 minutes of which the initial 10 minutes are used for rinsing the mucosa and for obtaining stable absorption conditions Four blood samples are drawn from the ear at intervals of five minutes (10 15 20 and 25 minutes after start of the perfusion) During this period the plasma salicylate consentration plotted against time gives a straight line The slope (α) of this line is used as a measure of the absorption rate During the perfusion it is possible to change to another perfusate and to determine the effect of an altered composition of the solution

In this type of experiment the conditions are well defined in contrast to the re circulation type where e g the concentration of the substance in the solution decreases gradually and where correction for substance accumulated in the human intestinal tissue is not possible

Discussion

With the technique described it is possible to study the absorption from a limited part of the rectum, which is kept constantly in contact with a well defined solution

The ideal conditions put forward in the introduction are to a great extend fulfilled, although some training is necessary to master the drainage

The technique has proved to be useful in absorption studies (BECHGAARD 1973) in which the absorption rate of salicylic acid is found to be proportional to the concentration and influenced by the pH in the perfusion solution Some of the experiments were performed by perfusing 2 cm, 4 cm or 7 5 cm of the rectum As might be expected the absorption increases with the length of the perfused rectal segment

Primarily the information obtained from rectal perfusion is usable in rectal drug administration Furthermore the results may give a hint as to the conditions of the biological membranes, especially the mucosa of the colon

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Absorption of Salicylic Acid from the Perfused Human Rectum

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Abstract The absorption of salicylic acid from solutions in which it is highly ionized was investigated by means of a new *in vivo* perfusion technique in human subjects The absorption rate shows proportionality to the concentration (15 6 mM–250 mM) while the rate decreases with increasing pH (5 9–8 9) This is in agreement with the theory of passive diffusion of the unionized form of the drug From a 47 mM sodium salicylate solution buffered to pH 7 9 with phosphate 7 5 cm of rectum absorbs about 100 mg per hour When a glycine buffer is used instead of the phosphate buffer the absorption rate increases about 2 5 times

Key words Rectal absorption – salicylic acid – perfused man

The absorption of weak organic acids or bases from the alimentary tract has been especially investigated by the BRODIE group (e g SCHANKER 1971) It is generally accepted that these compounds are mainly absorbed by penetration of the unionized form of the drug through a lipoidal barrier However, it is still an open question, to what extent the ionized form is able to pass membranes, and to what extent the pH partition theory is quantitatively valid

It has been reported that the ionized form of some drugs is probably absorbed from the small intestine KAKEMI *et al* (1969) conclude from *in vivo* experiments in rats that e g ionized salicylic acid is absorbed to a great extent in the small intestine at a rate depending on its binding to the mucosal surfase TURNER *et al* (1970) have shown *in vitro* that e g salicylic acid, when highly ionized, has a greater permeability constant from the mucosal side than from the serosal side of the rat intestine JACKSON *et al* (1970) conclude from *in vitro* experiments that the rat jejunum possesses a specific mechanism for the transport of some aromatic acids The *in vitro* experiments of NOGAMI *et al* (1961) on the rat small intestine have

shown that the permeability of unionized salicylic acid is only 6 times higher than that of the ionized form. CROUTHAMEL *et al.* (1971) find that the permeability ratio is about 5 for sulphaethidole in *in vivo* experiments on rats.

These findings are not in agreement with the results obtained by HOGBEN *et al.* (1959), who examined the steady state distribution of drugs across the rat intestine *in situ*. Assuming that the pH at the membrane in the small intestine is 5.3, they calculated the permeability of unionized salicylic acid to be 4500 times higher than that of the ionized form. However, salicylic acid has often been found to be amazingly well absorbed even at a pH in which it is highly ionized (e.g. DOLUISIO *et al.* 1969).

The present paper presents results from experiments using a procedure for human rectal perfusion (BECHGAARD 1973), in which the influence of luminal concentration and pH on the absorption rate has been investigated.

Methods

Procedure of absorption experiments

The perfusion of the rectum was carried out on healthy male students and the absorption was determined in the blood as described by BECHGAARD (1973). A buffered sodium salicylate solution was perfused at a rate of 6 ml per minute. At this perfusion rate the concentration of salicylate in the rectum was considered constant, the concentration in the effusate being nearly the same as that in the infusate. The pH of the urine was kept below 6 by the administration of 0.5 g ammonium chloride 1–2 hours before the experiment. In this way the elimination rate was decreased. As the experimental period was short and as the experimental results were meant to be compared with each other, the elimination was therefore disregarded in the calculations.

Perfusion solutions

Solutions used for the investigation of the influence of the concentration on the absorption rate were buffered with phosphate to pH 6.5 (buffer capacity (B) = 0.03). The salicylate concentrations varied from 15.6 mM to 250 mM. Buffered solutions with concentrations higher than 50 mM were hypertonic. Weaker solutions were made isotonic by the addition of sodium chloride.

In the experiments in which the influence of pH on the absorption rate was examined, the solutions were isotonic and contained sodium salicylate in a concentration of 47 mM. Phosphate buffers were used for pH 5.9 (B=0.013), pH 6.9 (B=0.06) and pH 7.9 (B=0.01). As the pH in the rectum is reported to be about 7.9 (BITTERMAN *et al.* 1967) it might be assumed that the buffer capacity of solutions at this pH does not need to be high. At pH 8.9 the phosphate buffer has too little capacity. Hence a glycine buffer (B=0.055) was used at this pH. Furthermore a glycine buffer pH 7.9 (B=0.01) was used.

Analytical methods

Salicylic acid was determined in the plasma in the same way as described for p-aminosalicylic acid by RASMUSSEN (1968). The fluorescence was read at pH 11 on an

Aminco-Bowman spectrofluorometer at 410 mμ, activated at 300 mμ Plasma standards and blanks were run through the procedure In the experiment where salicylic acid was analysed separately, apart from salicyluric acid the fluorescence was read at pH 5.5 instead of at pH 11 (SCHACHTER *et al* 1958)

Results

The influence of the concentration on the absorption rate

Fig 1 shows that direct proportionality is found between the absorption rate and the concentration of salicylic acid in solutions with pH 6 5 It is also seen that the absorption increases with the length of the perfused rectal segment

Fig 2 is an example of an experiment during which the concentration of salicylic acid is changed from 15 6 mM to 156 mM It demonstrates that a ten fold increase in the concentration gives a ten fold increase in the absorption rate

The results indicate that the absorption of salicylic acid shows no sign of limitation even at concentrations as high as 250 mM

The influence of pH on the absorption rate

The ratio between the absorption rates from phosphate buffers pH 7 9, 6 9 and 5 9 is found to be 1 2 3 (fig 3), which can also be demonstrated in a single experiment (fig 4) With the glycine buffer the absorption rate is

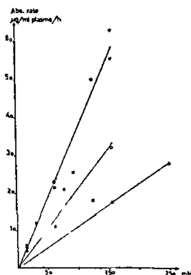


Fig 1 The influence of the concentration on the absorption rate of salicylic acid (pH 6 5) Length of perfused segment ● 7 5 cm ○ 4 cm ▲ 2 cm

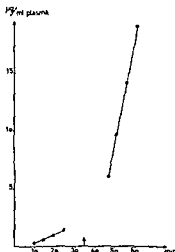


Fig 2 The absorption of salicylic acid during successive perfusion of two different concentrations ● 15 6 mM salicylate (pH 6 5) ○ 156 0 mM salicylate (pH 6.5) ↑ Change of perfusion solution

increased to about 2 5 times the rate found with the phosphate buffer at pH 7 9 Furthermore, fig 3 shows that the absorption is still pH dependant at pH 8 9, because it is smaller than the absorption rate from both the phosphate buffer and the glycine buffer at pH 7 9

The pH in the rectum was measured immediately before the experiment with a Radiometer GK 2302 C combined electrode 6–7 cm from the outer opening of the anal canal the rectal pH was normally found to be 7 6–7 8 The pH was remeasured 2 minutes after the perfusion However, the post perfusion regulation of rectal pH is about 0 5 pH unit per minute, with the consequence that the pH almost returned to normal 2–3 minutes after the experiment In spite of the alkaline reaction of the rectum the pH in the perfusate never changed by more than 0 1 unit at the perfusion rate used

The disappearance of salicylic acid from the plasma in 5 subjects, followed during one hour after the experiment, was 25–30 % per hour As the distribution space in man is about ten liters it is possible roughly to calculate that the 7 5 cm of rectum used absorb about 100 mg salicylic acid per hour from a 47 mM solution buffered with phosphate to pH 7 9

Discussion

The results are in agreement with those obtained by Schanker (1959) by the *in situ* rat colon perfusion They found that the absorption was proportional to the concentration and dependent on the pH in the perfusion solu

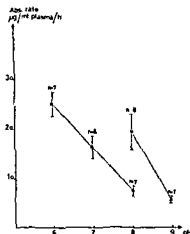


Fig 3 The influence of the pH on the absorption rate of salicylic acid (47 mM)
● Phosphate buffer
× Glycine buffer
Absorption rate Mean ± S E M

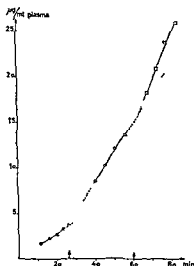


Fig 4 The absorption of salicylic acid during successive perfusion of three solutions (47 mM) buffered with phosphate to different pH
● pH 7 9
○ pH 6 9
□ pH 5 9
↑ Change of perfusion solution

tion, and suggested that drugs in the colon are passively absorbed in the unionized form across a barrier which is lipoidal in character like the barriers in the small intestine and in the stomach

KAKEMI *et al* (1969) also observed that the absorption of salicylic acid in the small intestine is influenced by pH in the pH interval in which salicylic acid is highly ionized In contrast to the absorption rate found in the colon and rectum they considered that their results were in better agreement with the binding of salicylate to isolated mucosa than with the dissociation curve of salicylic acid

However it is theoretically possible to explain even the pH dependant absorption results of KAKEMI *et al* as a diffusion of the unionized form Calculations from the Henderson Hasselbalch equation show that the concentration of unionized salicylic acid (pKA = 3) increases ten times when the pH in the solution is lowered from 8 to 7, and 100 times when the pH is lowered from 8 to 6 Consequently the theoretical ratio between the absorption rates should be 1 10 100 The relation found is 1 2 3, i e of the same magnitude as is seen in perfusion experiments on rats (e g KAKEMI *et al* 1969, HOGBEN *et al* 1959) This is far from the theoretical ratio One explanation for this discrepancy may be that it is difficult to change the pH at the absorbing membrane The results of corresponding experiments on a 25 μm thick artificial lipoidal membrane (DIBBERN *et al* 1969, fig 2 3 5) are likewise inadequately described by the Henderson-Hasselbalch equation The Henderson Hasselbalch equation is a static model, which does not take into account structural changes of the membrane and kinetics Experiments on artifical membranes might be used as a supplement to the equation to express the theoretical influence of pH on the absorption rate

It is possible that the ionized form is bound to a substance in the mucosa and in this way transported through the membrane This mechanism would be expected to have a limited capacity but the absorption rate at pH 6 5 is found to be proportional to the concentration of the perfusate up to 250 mM (4 % sodium salicylate solution) and possibly even stronger

TRAVELL (1960–61) found that salicylic acid accelerates the absorption of strychnine from the stomach of cats and concluded that salicylic acid destroys the epithelial barrier She suggested that epithelial destruction might explain why other investigators found highly ionized salicylic acid to be well absorbed from the small intestine KUNZE *et al* (1972) suggested that salicylate interacts with bivalent metals in or at the epithelial cells, with the result that the membrane permeability is increased Fig 2 shows that during an experiment the absorption rate from the rectum is constant Therefore there is no indication that salicylic acid gradually influences the permeability of the absorbing membrane In any case destruction must have been completed within the initial 5–10 minutes of the perfusion before the period

of blood samplıng In addıtion proportıonalıty ıs found between the concentratıon and the absorptıon rate A ten fold ıncrease ın the concentratıon results ın a ten fold ıncrease ın the amount absorbed An ıncreased permeabılıty of the membrane ıs most lıkely to a certaın extent dependent on the concentratıon If the permeabılıty e g ıs ıncreased two tımes by raısıng the concentratıon ten tımes, the absorptıon ın the above mentioned example should ıncrease twenty tımes ınstead of the ten tımes observed

The fact that salıcylıc acıd ıs well absorbed from a lumınal pH ın whıch ıt ıs hıghly ıonızed, ıs dıffıcult to explaın accordıng to the pH partıtıon theory However, as a small fractıon of salıcylıc acıd ıs always present unıonızed, the reversıble dıssocıatıon beıng an almost ınstant process, the results obtaıned could explaın the absorptıon of salıcylıc acıd from the human rectum as a passıve dıffusıon of the unıonızed form

The ıncreased absorptıon rate from the glycıne buffer compared wıth the phosphate buffer ıs a separate problem It ıs possıble that glycıne and salıcylıc acıd are conjugated, and thus absorbed as the metabolıte salıcylurıc acıd, but ıf salıcylıc acıd ıs determıned when ısolated ın the blood, an ıncreased absorptıon rate ıs stıll found (fıg 5) Thıs makes the assumptıon of absorptıon as the metabolıte unlıkely

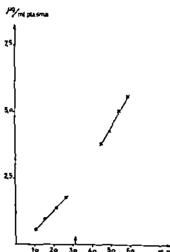


Fıg 5 The absorptıon of salıcylıc acıd durıng successıve perfusıon of two solutıons (47 mM) wıth dıfferent buffer systems

- ● Phosphate buffer pH 7 9 (buffer capacıty 0 01)
- × Glycıne buffer pH 7 9 (buffer capacıty 0 01)
- ↑ Change of perfusıon solutıon

In conclusion salicylic acid, although highly ionized in the perfusion solution, is well absorbed from the human rectum. It is possible to describe this absorption as a passive diffusion of the unionized form through a barrier, which is lipoidal in character.

Though physiological factors may decrease the influence of pH on the absorption rate, this cannot account for the fact that the effect observed is much smaller than expected from the Henderson-Hasselbalch equation. Results from equivalent experiments on artificial membranes may be a valuable addition in theoretical calculations.

It is most likely that glycine increases the absorption of salicylic acid.

The permeability of the absorbing surface is probably not influenced by salicylate during the experiment If any, this influence must be completed in the initial 5–10 minutes of the perfusion.

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Biological Availability and *in Vitro* Release from Oral Oxytetracycline and Tetracycline Preparations

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Abstract The *in vitro* dissolution rates of 7 oxytetracycline and 2 tetracycline preparations and the absorption characteristics as obtained by a randomized cross over study on 10 healthy volunteers were studied It was found that a Two oxytetracycline preparations (nos 1 and 7) were inferior to the others in terms of mean serum levels and in producing non detectable or insufficient serum concentrations in 2 and 3 persons respectively b The bio-availability of the 5 other oxytetracycline preparations were of the same order (statistically not significantly different) although one preparation (no 3) exhibited higher mean serum levels c The area under the serum concentration curves were proportional to and correlated with the amounts of oxytetracycline and tetracycline recovered in the urine during the period of study d The rate of dissolution was related to the absorption characteristics cfr the preparations 1 and 3 No correlation could be demonstrated between the results of the dissolution tests and the results of the absorption study for preparation 7 e The tetracycline preparations gave serum concentrations above those of most oxytetracyclines f The oxytetracycline and tetracycline preparations tested showed marked variation in the *biological availability of active ingredients*

Key words Tetracycline – oxytetracycline – bioavailability – *in vitro* release

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Differences in pharmaceutical formulations of generic preparations may significantly influence the rate and extent of absorption from the gastrointestinal tract (AGUIAR *et al* 1968, BELL *et al* 1971, CASTLE *et al* 1969, LEVY & NELSON 1961, WAGNER 1961, WAHLQVIST 1969)

For antibacterial therapy, it is essential that the antibiotic concentrations at the focus of infection are maintained above certain thresholds determined by the sensitivity of the infectious agent Differences in biological availability for antibiotics may have serious consequences

In 1969, the Food and Drug Administration (FDA) withdrew its certifica tion for oxytetracycline preparations from 10 producers pending the demonstration of adequate serum levels comparable to a reference product (terramycin® »Pfizer« capsules) (BLAIR *et al* 1971, BRICE & HAMMER 1969, EDITORIAL 1970, BARR *et al* 1972) By the end of 1969, only two manufacturers managed to present satisfactory evidence

Before 1969/1970, the Norwegian health authorities required complete documentation for preparations containing new active substances before these were brought on the market Subsequent applications of equivalent drugs were, however, accepted on the assumption that generic identity also indicated therapeutic equivalence, and investigations on serum concentrations were not required for these products All oxytetracycline preparations on the Norwegian market were introduced before 1969/1970 Accordingly, an examination of the biological availability of these preparations, and, for comparison, some tetracycline preparations was deemed desirable

The absorption characteristics for the tetracycline/oxytetracycline preparations on the Norwegian market have been amply demonstrated for the tetracycline aureomycin® »Lederle« and oxytetracycline terramycin® which have been used in several extensive comparisons with other formulations (BARR *et al* 1972, BLAIR *et al* 1971, BRICE & HAMMER 1969 MACDONALD *et al* 1969) The absorption characteristics of oxy Dumocyclin® »Dumex« have also been reported (HANSEN 1967) No published absorption data are available for the other marketed tetracycline or oxytetracycline preparations

The present communication presents a cross-over absorption study on the 7 available oral oxytetracycline preparations and 2 tetracycline formulations *In vitro* analyses of disintegration and dissolution were carried out on the same batches that were included in the absorption study

Materials and Methods

Drug preparations

All the 7 marketed oral oxytetracycline (OT) drugs from 5 producers (as of May 1 1972) (oxytetracycline is the most widely used tetracycline derivative in this country accounting for 78 per cent of the total number of doses of tetracyclines sold (HALSE M personal communication 1972)) and two tetracycline (T) formulations were examined The terramycin® capsules and the mixture (preparations nos 6 and 4) and achromycin® capsules (preparation no 8) were considered as standards as these were the first generic preparations marketed Preparations nos 3 and 5 were the tablets and mixture respectively from the same producer

All the preparations examined were commercial batches obtained directly from the Norwegian state monopoly for drugs (Norsk Medisinaldepot – NMD) This ensured that

Table 1

Products included in the study

Preparation* number	Declared quantity per dose (as oxy tetracycline (OT) or tetracycline (T) base) (g)	Pharmaceutical dosage form	Lot number	Expiration date	
1	0 250 OT***	Sugar coated tablets	10203	1 February	1974
2	0 233 OT****	Sugar coated tablets	9/74/G	September	1974
3	0 250 OT****	Film coated tablets	18206	1 October	1976
4	0 250 OT**	Mixture	101 50252	March	1975
5	0 250 OT**	Mixture	17231	1 November	1973
6	0 250 OT****	Capsules	101 51603	January	1976
7	0 280 OT****	Sugar coated tablets	35181	January	1973
8	0 228 T****	Capsules	4768-07 8250	31 May	1975
9	0 228 T****	Capsules	8973	June	1975

* Preparations 1 and 7 were withdrawn from the Norwegian market in May 1972
** The OT base concentration in the solutions were 25 mg/ml (as calcium salt)
*** OT as dihydrate
**** OT and T as chloride.

the preparations had been stored under recommended conditions and were the same as those delivered for sale at this time

Subjects

Ten healthy medical student volunteers participated in the study (table 2) Any other medication during the period of the investigation was not allowed

Table 2

Characteristics of test subjects

Subject no	Sex	Age	Weight kg
1	M	27	66
2	M	23	68
3	M	22	78
4	M	24	77
5	M	25	77
6	M	22	73
7	M	28	75
8	M	23	67
9	M	22	70
10	F	30	55

Table 3

Evaluation of assay accuracy *

Variable designation		Numerical values		
Intended specimen concentration	(µg/ml)	0 45	0 90	1 40
Measured mean	(µg/ml)	0 456	0 828	1 248
Relative standard deviation (S D)	(%)	8 36	6 67	8 42
Highest measured value	(µg/ml)	0.54	0 95	1.50
Lowest measured value	(µg/ml)	0 38	0 72	1 05
The deviation of the mean from intended concentration	(%)	1 3	8 0	10 9
Largest deviation from the intended concentration	(%)	16 7	20 0	25 0

* As a basis for the calculations three "unknowns" made up to contain concentrations of 0 45 0 90, and 1 40 µg/ml oxytetracycline-HCl were measured 28 times each For this purpose, 11 holes were included on each of the triplicate plates each with its own standard set (individual standard curves for the triplicate with 0.5, 1 0 2 0, 4 0 and 8 0 µg/ml) and 6 positions for the "unknowns" Each of the 28 × 3 determinations was thus the mean of three observations (i e on 3 parallel plates 6 unknowns' could be assayed)

Examination procedures

The subjects had been fasting over night and were allowed to eat only 3 hours after medication

Doses of one tablet or capsule or the equivalent quantity of the mixtures were given The declared OT and T quantities per dosage unit appears in table 1 The drugs were swallowed with one glass of water

Usually, one week elapsed between tests but once there was only a 3 day and once a 4 day interval Venous blood was withdrawn at 1, 2 3, 4 and 6 hours after drug intake by indwelling venous catheters (Venflon®) Urine samples were collected during the 6 hours of the study Serum and urine samnles were frozen to −70° within 1 hour and analysed within 3–5 days

Microbiological assay

Staphylococcus aureus ATCC 6538p was used as the indicator organism in an agar cup technique on Mueller Hinton Medium (Difco) agar similar to that previously described (BERGAN & ØYDVIN 1972)

Serum standards and dilutions were made with pooled human serum For urine, the diluent was Sørensen s phosphate buffer of pH 7 0 The most useful assay interval was 0 2–10 0 µg/ml the lower limit of sensitivity being approximately 0 1–0 15 µg/ml Evaluations of assay reproducibility appear in table 3

Reagents

The antibiotic reference substances were obtained from stock at NMD The oxytetracycline chloride was from lot A nr 0C 151 Quantitation according to the British Pharmacopeia showed 101.3 per cent specific substance *The tetracycline chloride*

Table 1

Products included in the study

Preparation* number	Declared quantity per dose (as oxy tetracycline (OT) or tetracycline (T) base) (g)	Pharmaceutical dosage form	Lot number	Expiration date	
1	0 250 OT***	Sugar coated tablets	10203	1 February	1974
2	0 233 OT****	Sugar coated tablets	9/74/G	September	1974
3	0 250 OT****	Film coated tablets	18206	1 October	1976
4	0 250 OT**	Mixture	101 50252	March	1975
5	0 250 OT**	Mixture	17231	1 November	1973
6	0 250 OT****	Capsules	101 51603	January	1976
7	0 280 OT****	Sugar coated tablets	35181	January	1973
8	0 228 T****	Capsules	4768 07 8250	31 May	1975
9	0 228 T****	Capsules	8973	June	1975

* Preparations 1 and 7 were withdrawn from the Norwegian market in May 1972
** The OT base concentration in the solutions were 25 mg/ml (as calcium salt)
*** OT as dihydrate
**** OT and T as chloride

the preparations had been stored under recommended conditions and were the same as those delivered for sale at this time

Subjects

Ten healthy medical student volunteers participated in the study (table 2) Any other medication during the period of the investigation was not allowed

Table 2

Characteristics of test subjects

Subject no	Sex	Age	Weight kg
1	M	27	66
2	M	23	68
3	M	22	78
4	M	24	77
5	M	25	77
6	M	22	73
7	M	28	75
8	M	23	67
9	M	22	70
10	F	30	55

Table 4

Disintegration time and quantitation of dosage units

Characteristic	Method of analysis	Denomination	Preparations 1	2	3	4	5	6	7	8	9
Quantitative assay of dose units	Ph Nord 1963*	Per cent of amount declared per dosage unit	96 9	98 0	105 1	99 5	97 1	97 1	100 9	100 1	101 3
Disintegration	Ph. Nord 1963	minutes	30	20	< 1	–**	–	6	10	10	5
	USP XVIII***	minutes	30	16	< 1	–	–	8	8	4	3

* Pharmacopoea Nordica 1963
** – = non evaluable
*** United States Pharmacopeia, vol 18

Table 5

Time of 50 per cent dissolution for tablets and capsules of oxytetracycline and tetra cycline preparations belonging to the batches used in cross-over absorption studies

Preparation	Times of 50 per cent dissolution (min)	Mean	Standard deviation
1	ND*, ND, ND 52 0, 12.5, 12 0, 15 0	NC	NC**
2	7.5 5 8 5 6 7 5, 6 8, 5 5, 15 0	7 7	3 3
3	Between 0 and 1 minute	<1	NC
6	6 2, 7 2 6 4, 6 4, 6 4, 23 0	9.3	6 7
7	14 0 5 9, 4 5, 6 7, 8 0, 7 1	7 7	3 3
8	5 6, 4 0, 4 0 4.3, 4 2, 4 6	4 5	0 6
9	4 2, 3 5, 2 6, 2 9, 3 8	3 4	0 7

* ND = 50 per cent dissolution not reached within 60 minutes

** NC = not calculable due to particularly long or short (i e without numerical characterization) dissolution times

Preparation no 3 dissolved completely (> 95 %) within 2 minutes *and* was thus superior to all the other preparations tested The dissolution characteristics for preparations 2, 6 and 7 were fairly similar to each other, one tablet of each showing a retarded dissolution

Three of the tablets from preparation 1 failed to disintegrate The coating of four other tablets from this preparation were divided into two parts without further disintegration during 60 minutes The active ingredients in three of these tablets were almost completely dissolved within 30 minutes and in the fourth within 60 minutes All the tablets of preparation 7 exhibited hard

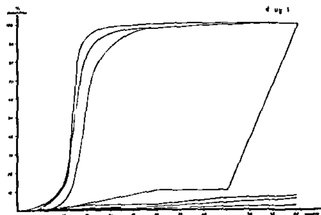


Fig 1 Dissolution curves for individual tablets of preparation number 1

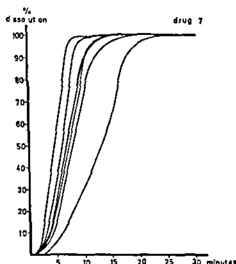


Fig 2 Dissolution curves with individual tablets of preparation number 7

fragments of coating which did not further disintegrate Similar observations were not made for the other preparations

Biological availability

The results of the absorption studies are detailed in figs 4–9 All the individual results are given so as to document the details of the differences

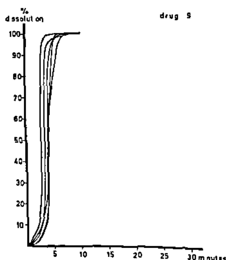


Fig 3 Dissolution curves of individual capsules of preparation number 9

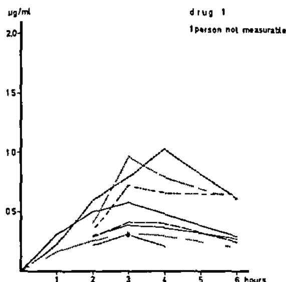


Fig 4 Serum concentrations reached with the oxytetracycline preparation number 1 in 10 individuals When only one measurable serum concentration was found for an individual, this is indicated with a filled dot (●)

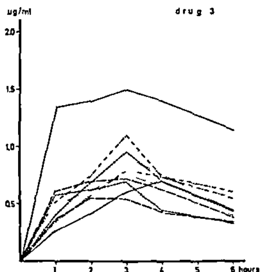


Fig 5 Serum concentrations reached with the oxytetracycline preparation number 3 in 10 individuals

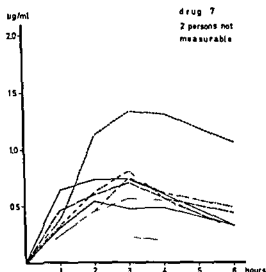


Fig 6 Serum concentrations obtained with the oxytetracycline preparation number 7 in 10 individuals Legend see fig 4

in serum concentrations reached with different dosage units of the drugs Table 6 shows the standard deviations and range of values observed at each sampling time The mean concentration curves for the 7 oxytetracyclines appear in fig 8 and for the two tetracyclines in fig 9 The values are given as measured, without numerical adjustments for differences in dosage size

The mean peak serum concentrations of the T and OT preparations were always reached after 3 hours The highest mean peak concentration obtained was approximately 1 µg/ml with the oxytetracycline preparation no 3 and the tetracycline 9

Preparations 1 and 7 demonstrated the least favourable characteristics, these had the lowest mean serum concentrations (figs 4 and 6) The differences between the serum concentrations of preparation 1 and 3 (the latter having the highest mean concentrations) was highly significant at all sampling times ($P < 0\,001$) The differences between preparations 3 and 7 were also highly significant It should be noted, however, that the declared OT content of preparation 7 was approximately 12 per cent higher than that for preparation 1 A considerable variation in the biological availability of preparation 1 was observed One of the tablets tested failed to give any demonstrable serum concentrations, another tablet showed measurable amounts only at the 3 hour sampling With preparation 7, measurable serum concentrations were not obtained in two of the subjects and in a third subject low levels were obtained only at the 3 and the 4 hour samplings

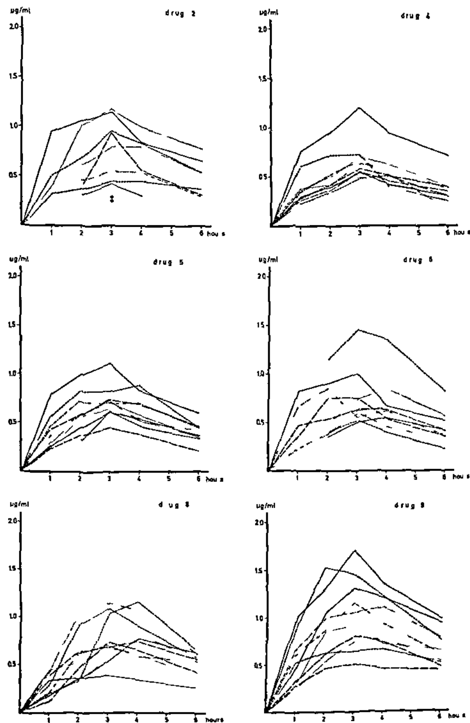


Fig 7 Serum concentrations reached with the oxytetracycline preparation numbers 2, 4, 5 6 and the tetracycline drugs 8 and 9 Legend see fig 4

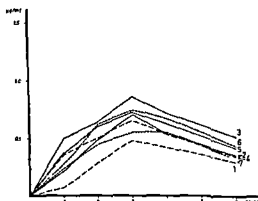


Fig 8 Mean serum concentrations after one dose of the oxytetracycline preparations 1 to 7 The variation on each sampling appears in table 6

The remaining OT preparations, 2, 4, 5 and 6, had lower mean values than preparation 3 which exhibited the highest mean peak value However, statistically, the four former preparations were not *significantly different* from preparation 3 It should be noted, however, that preparation 2 in two of the ten test subjects only showed detectable serum concentrations at the 3 hour sampling

Individual variation of absorption

Drug absorption may differ from one subject to another Likewise, one may find different responses on repeated tests in the same subjects It is consequently of interest to monitor the extent of individual variation

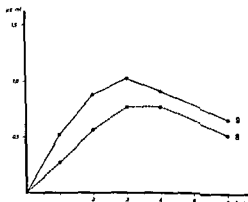


Fig 9 Mean serum concentrations for the tetracycline preparation numbers 8 and 9 The variation on each sampling is reported in table 6

Table 6

Range (R) and standard deviations (S D) of serum concentrations after 7 oxytetra cycline and 2 tetracycline preparations

Prepara tion	Variable	Hours after drug intake 1	2	3	4	6
1	R	0–0 23	0–0 60	0–0 97	0–1 03	0–0 65
	S D	0 119	0 191	0 284	0 331	0 258
2	R	0–0 96	0–1 07	0 29–1 18	0–1 00	0–0 78
	S D	0 327	0 367	0 350	0 350	0.285
3	R	0–1 35	0–1 40	0 55–1 50	0 45–1 40	0 33–1 15
	S D	0 347	0 344	0 278	0 266	0 240
4	R	0–0 76	0 34–0 95	0 48–1 20	0 39–0 95	0 17 0 71
	S D	0 205	0 192	0 208	0 164	0 143
5	R	0–0 80	0 32–1 00	0 45–1 12	0 38–0 47	0 21–0 61
	S D	0 217	0 207	0 175	0 164	0 106
6	R	0–0 82*	0 35–1 15	0 50–1 45	0 39–1 35	0 31–0 81
	S D	0 315*	0 277	0.291	0 271	0 164
7	R	0–0 64	0–1 12	0–1 32	0–1.30	0–1 05
	S D	0 228	0 364	0 220	0 375	0 312
8	R	0 12–0 44	0 29–1 05	0 39–1 10	0 35–1 17	0 26–0 66
	S D	0 121	0 260	0 252	0 240	0 128
9	R	0 24–1 00	0 45–1 52	0 50–1 70	0 45–1 35	0 43–0 97
	S D	0 263	0 347	0 380	0 293	0 189

* This figure calculated from 9 blood samples

To this end, the results after giving the mixtures, preparations 4 and 5, are relevant With these the process of disintegration is omitted It is also seen that the serum concentrations with nos 4 and 5 showed little variation as compared with the tablets or capsules

Urinary excretion

As is apparent from table 7, urinary recovery was mostly correlated to absorption as evaluated from the size of the areas under the curves (AUC) for the serum concentrations Fig 10 shows examples of these relationships for the preparations 1 and 7 The subjects with the highest peak serum concentrations tended to have the largest AUC's

The table 8 shows the relative AUC s and amounts recovered in the urine for the OT preparations The higher serum concentrations with drug no *3 is* reflected in lower values in the rows and higher numbers in the columns of the table for the relative AUC-values and amounts of OT recovered The good agreement between AUC and the amounts recovered is reflected by the correlation coefficient $r = 0\,7264$ ($P < 0\,001$) for the 84 entries of the

Table 7

Percentage of oral doses of oxytetracycline (preparations 1–7) and tetracycline (nos 8 and 9) preparations recovered in the urines (%) and areas under the serum concentration curves (AUC)

Preparation	Variable	Test individuals 1	2	3	4	5	6	7	8	9	10	Mean	S D *	r**	P***
1	%	14.3	7.4	21.4	8.1	7.3	16.3	4.9	23.0	2.3	5.4	11.0	7.2	0.8367	< 0.001
	AUC	2.63	2.30	3.06	0.85	1.52	1.39	0.30	3.70	0	1.44	1.72	1.19		
2	%	11.4	8.1	20.2	5.9	23.0	5.6	10.5	32.0	23.5	10.1	15.0	9.0	0.6544	0.01 < P < 0.05
	AUC	2.06	1.15	3.80	0.25	2.34	0.28	2.11	4.68	3.08	5.04	2.53	1.78		
3	%	19.2	14.3	18.9	15.9	12.4	21.0	12.2	20.4	22.3	23.8	18.0	6.9	0.7118	< 0.01
	AUC	3.93	2.76	3.17	2.84	2.46	3.41	2.46	3.57	3.33	7.41	3.49	1.52		
4	%	18.7	18.2	17.0	16.1	11.8	16.2	13.6	14.7	13.6	20.7	16.1	2.7	0.6657	0.01 < P < 0.05
	AUC	2.67	2.16	2.86	2.07	2.14	2.54	2.25	2.91	2.25	5.06	2.69	0.89		
5	%	19.6	12.3	11.8	13.6	17.8	17.6	17.1	23.1	19.3	18.9	17.1	3.6	0.5406	> 0.05
	AUC	2.90	2.54	2.65	1.98	1.83	3.44	3.31	4.06	2.82	4.91	3.04	0.93		
6	%	10.6	12.6	14.2	10.2	12.7	16.2	11.3	25.2	15.4	11.4	14.0	4.4	0.7450	< 0.01
	AUC	2.19	2.09	3.04	1.72	3.02	3.40	3.03	5.48	2.42	4.28	3.03	1.12		
7	%	17.5	12.6	16.6	1.2	13.4	21.4	0.8	22.4	5.7	15.9	12.8	7.8	0.8957	< 0.001
	AUC	2.64	2.43	2.37	0	3.16	2.95	0	5.94	0.51	3.43	2.34	1.81		
8	%	25.9	15.6	29.1	20.7	24.1	24.3	36.8	29.3	24.6	7.0	23.7	8.1	0.5244	> 0.05
	AUC	4.95	2.85	3.04	4.00	2.73	2.93	3.47	4.33	2.83	1.90	3.30	0.90		
9	%	27.6	24.7	25.5	31.2	24.3	26.8	26.8	38.8	24.5	33.9	28.4	4.8	0.6278	0.01 < P < 0.05
	AUC	5.06	5.49	3.74	3.35	3.34	4.63	2.32	6.35	3.18	6.94	4.44	1.50		

* S D = standard deviation

** r = correlation coefficient.

*** P = probability of correlation (evaluated by $t = r\sqrt{n-2} / \sqrt{1-r^2}$)

Table 8

Relative mean areas under the curves (AUC) and percentages of actual dose recovered in urine (%) for the 7 oxytetracycline preparations

A AUC_x/AUC_y

Preparations as denominators	Preparations as numerators 1	2	3	4	5	6	7
1	–	1.471	2.029	1.564	1.767	1.762	1.360
2	0.680	–	1.379	1.063	1.202	1.198	0.925
3	0.493	0.725	–	0.771	0.871	0.868	0.670
4	0.616	0.907	1.254	–	1.130	1.126	0.870
5	0.566	0.832	1.148	0.885	–	0.997	0.770
6	0.568	0.835	1.152	0.888	1.003	–	0.772
7	0.735	1.081	1.491	1.150	1.299	1.295	–

B $\%_x/\%_y$

Preparations as denominators	Preparations as numerators 1	2	3	4	5	6	7
1	–	1.364	1.464	1.464	1.555	1.273	1.164
2	0.733	–	1.073	1.073	1.140	0.933	0.853
3	0.683	0.932	–	1.000	1.062	0.870	0.795
4	0.683	0.932	0.932	–	1.062	0.870	0.755
5	0.643	0.877	0.942	0.942	–	0.819	0.749
6	0.786	1.272	1.150	1.150	1.221	–	0.914
7	0.859	1.172	1.158	1.258	1.336	1.094	–

The correlation coefficient for table A versus table B is $r = 0.7264$ which corresponds to correlation probability of $P < 0.001$

bodies of the table 8 The AUC for preparation 3 is 2.029 times larger than the AUC for preparation 1 and 1.491 times that of no 7 The corresponding figures for the amounts recovered in the urine are 1.464 and 1.158 These data correlate well with the highest mean serum concentrations for preparation 3 and the lowest for no 1

Discussion

These absorption studies revealed that the different OT and T preparations tested differed in their biological availability Preparation 1 and 7 showed serum concentrations which at peak values were markedly lower than preparation 3 ($P < 0.001$), the dissimilarities were also significant at other times

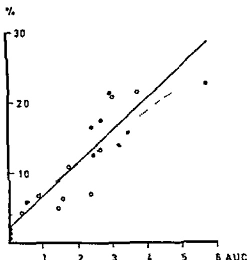


Fig 10 Interdependence for the area under the curve (AUC) and per cent specific substance recovered in the urine (%) during 6 hours for the oxytetracycline preparations 1 and 7

of sampling Although preparation 3 gave higher mean serum concentrations than the other preparations, the difference between the results obtained with this preparation and with preparations 2, 4, 5 and 6 were not statistically significant The formulations 1 and 7 showed both lower AUC's and less urinary OT recovery (tables 7 and 8)

In the present studies, single tablets or capsules have been given to detect differences in biological availability

Obviously, differences in serum concentrations after individual dosage units are more significant if absorption varies within fairly narrow limits The fact that absorption as such was a relatively stable event is demonstrated by the serum concentrations obtained with the liquid preparations 4 and 5 (fig 7), in which the bio availability was independent of a disintegration procedure With the mixtures the variation in individual serum levels is less It is important to note that the mixtures gave acceptable serum levels in all the subjects tested

The better serum profiles observed in individual 10 may partly be ascribed to the lower body weight of this subject

The preparations with the lowest serum concentrations, also gave lower urinary OT and T values The general trend in table 8 towards lower index values in the rows and higher figures in the columns for preparation 3, points towards a formal relationship between the two variables Thus, when the serum concentrations with preparations 1 and 7 are low or non measurable, insignificant amounts are recovered concomitantly in the urine It can

be shown that the formal relationship between the serum concentrations and renal excretion can be expressed by the formula

$$M = Cl\ p\ \left(A_t + \frac{c_t}{k_2}\right)$$

Cl is renal clearance, p is the protein binding factor, A_t is area under the curve (AUC) during the finite time t of the observation period, c_t is the serum concentration at time t, and k_2 is the coefficient of excretion.

The overall relationship between AUC and OT recovery in the urine was demonstrated by the correlation coefficient $r = 0.7264$ ($P < 0.001$). This supports the above equation. The relative excretion was of the same order as the relative bio availability evaluated on the basis of AUC (table 8). Thus, once urine recoveries have been correlated to serum concentrations (2) recovery in the urine can be used as a preliminary value in routine controls of tetracycline preparations to monitor differences in bio availability.

The observations that there was little absorption and low urinary recovery for preparation 1 correlates well with the results of the pharmaceutical *in vitro* tests performed on the product. These findings show markedly slower and more varied dissolution rates for preparation 1 than for any of the other preparations. Out of 7 tablets tested, 3 only became soft without showing any dissolution, and one had a t_{50} as high as 52 minutes. Disintegration experiments (table 4) showed that preparation 1 disintegrated more slowly than all the other preparations. Some tablets only divided in two halves without any further disintegration. It seems likely that the two cases without any measurable serum concentrations were caused by tablets of inferior coating. Although the low bio-availability of preparation 7 is probably caused by the pharmaceutical formulation, the *in vitro* experiments offer no explanation, as the dissolution characteristics of these preparations did not differ significantly from those of preparation 2 and 6. The dissolution curves of the tablets from preparation 7 had practically the same course and spread as those of formulation 2 and 6. Nevertheless the difference between drugs 2, 6 and 7, namely that the preparations 2 and 6 gave measurable serum concentrations in all the test subjects, is a significant and important difference between these preparations.

It is important to note that preparation 1 with a low release also has a low bio-availability as compared to preparation 3, which showed the higher mean bio-availability and the more rapid dissolution. The correlation of rapid dissolution and better intestinal absorption has also been noted for other antibiotics, e. g. ampicillin (WAHLQVIST 1969) penicillin-V (JOHNSGÅRD & TORUD 1971, JUNCHER & RAASCHOU 1957) chloramphenicol (AGUIAR *et al.* 1968, GLAZKO *et al.* 1968), and sulfonamides (TARASZKA & DELOR 1969).

It is notable that the drug 9 exhibited higher serum concentrations than

the "reference standard" preparation 8 It is also notable that the tetracycline "standard' displayed the same levels of serum concentrations as the oxytetracycline "standard", 6 In the USA, preparation 6 showed a bio-availability superior to that of other generic preparations (BLAIR *et al* 1971, BRICE & HAMMER 1969), and has been used by the FDA as a reference product (EDITORIAL 1970)

Drug absorption involves many biological variables Accordingly, the serum concentrations recorded in this study are not definitive, since they may be related to the particular batches studied and the test subjects used The serum concentrations obtained for oxytetracyclines in this study are slightly lower than those found in other studies (BLAIR *et al* 1971, BRICE & HAMMER 1969), but the concentrations for tetracyclines tally with previous data (MACDONALD *et al* 1969) It is, however, not surprising that absolute figures may vary from one group of subjects to another Nevertheless, the relative values for the preparations should be valid Assay standards and assay procedure as well as the conditions and time of storage of the preparations before acquisition may also influence the results It should be noted that error in assay is the least for lowest concentrations, as the consequences are the greatest for drugs with the lowest serum concentrations Table 3, for instance, shows that the relative standard deviation for 28 determinations is only 1 3 per cent for a concentration of only 0 45 μg/ml Since this is of the same order as the peak serum concentrations measured for the preparations 1 and 7, the results and evaluations with these drugs should give the highest confidence

It is obvious that in many instances, more effort should be made in the pharmaceutical formulations of tetracyclines before they are certified for sale This entails that more basic studies should be performed in the first instance by the manufacturer

All the formulations should satisfy the requirements for quantitation, disintegration and dissolution set by the Ph Nord 1963 The requirements of Ph Nord 1963 which accepts 100 per cent disintegration after as much as 60 minutes for coated tablets is, however, entirely insufficient for tetracyclines The more realistic limits of 10 minutes have been set for capsules and non-coated tablets

The inadequate bio-availability of some of the OT formulations, obtained in the present study, could mostly have been avoided if requirements for the acceptance of new antibiotic formulations were rather rigid The results demonstrate the importance of testing pharmaceutical formulation *in vivo* *In vitro* tests (dissolution tests) do not always reveal inferior biological availability

It would be desirable in absorption studies to have a cross over randomized pattern using 10 or more subjects Comparison with such data should

make for more efficient and adequate information concerning the absorption of new products a) the new formulation (preferably of an age corresponding to the expiration date), b) a "reference" drug with known/certified bioavailability characteristics, and c) if possible a liquid preparation containing the same active ingredient as the formulation tested The purpose of the last control is to monitor the variability of absorption in the test subjects

The names of the preparations tested are available from the authors upon request Preparation no 1 has been reformulated and reappeared on the market

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Pharmacological Activity of *Paravespula Germanica* Wasp Venom

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Key words Wasp venom – *paravespula germanica* – toxicity

The wasp *Paravespula germanica* is prevalent in Euroasian countries, in North America, in North Africa and in New Zealand (LOKEN 1962, THOMAS 1960) In Israel it is found in the northern and central regions In the present paper we describe a number of pharmacological effects produced by the venom sac extract

Methods We collected approximately 6000 wasps, deep freezed them at – 20° and gradually extracted venom sacs from the insects by means of gentle pressure on the edge of the abdomen The venom sacs were homogenized extracted in distilled water, and then liophylized The dry substance was used for the experiments

The toxicity of the venom (venom sac extract) was determined by injection into the tail vein of male albino mice weighing 20 g The maximum volume injected was 0 3 ml Four groups of six animals each were used the final mortality being recorded 24 hrs after administration of the extract The LD_{50} was calculated by log probits method (FINNEY 1952) Within 1 hr the mice showed symptoms in the following sequence dyspnoea, akinesia, diarrhoea, mydriasis, loss of righting reflex, and paralysis This last mentioned symptom was of prolonged duration and the animals eventually died in coma The LD50 was 117 5 mg/kg The venom was dialysed for 24 hrs at 4° and injected into mice Moreover material retained in the dialysis sac produced the same effects as the whole solution and caused no lethal effects while the dialysate did not produce mortality even in triple doses Boiled venom (for 5') did not cause mortality at any dose level The venom was examined for protease and hyaluronidase activity by the methods of KOCHWA *et al* (1960) and DI-FERANTE (1956), respectively To determine haemolytic activity, drops of venom solution (100 µg/ml) were applied to Petri dishes containing agar human blood and incubated at 37° for 24 hrs It was found that the venom possessed protease and hyaluronidase activity and caused haemolysis Hista

mine releasing activity was tested in rat mast-cells obtained after intraperitoneal injection of buffer solution The cells were incubated with 150 μg (protein content) of venom for 45 min at 37° and then centrifuged After discarding the supernatant the cells were treated with 1 ml of 0 1 N-HCl and boiled for 3 min in order to release the remaining histamine The solution was then neutralized and tested on isolated guinea-pig ileum preparations No histamine remained in the mast cells after incubation with venom On the other hand, 50 mg of histamine could be liberated from the control cells at the end of the incubation period

Isolated guinea pig ileum and rat uterus were used to study the activity of venom solution on smooth muscles (EDERY *et al* 1972) Contractions of the ileum and uterus were recorded through a transducer on a polygraph (Grass) Doses of 0 35–0 75 mg venom caused slow, sustained contractions of both preparation Following the injection in the organ bath of 10 μg atropine sulphate (Mann), 1 mg mepyramine maleate (Teva) and 200 μg deseril® (Sandoz) (methysergidum NFN) before the venom injection, the contraction of both the organs tested was smaller

The activity of the venom on the cardiovascular system was examined

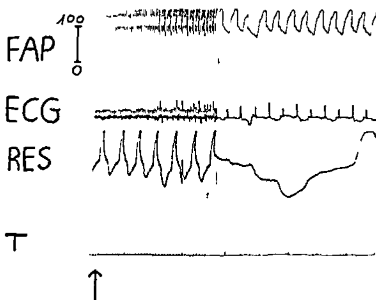


Fig 1 Ventricular bigeminy following injection of venom 4 mg venom sac extract after dialysis was injected (arrow) into the femoral vein of a cat of 3 0 kg Note that the blood pressure is in no way affected
FAP = femoral blood pressure
ECG = electrocardiogram lead II
RES = respiration

in six cats weighing 3-3 8 kg They were anaesthetized by intravenous injection of 25 mg/kg of sodium pentobarbital (mebumalum NFN) (nembutal®) (Abbott) and prepared for the recording of blood pressure from the femoral artery Simultaneously, an ECG was run in Lead II, as well as a respiration test The injection of 0 35–1 4 mg venom intravenously in the initial stage, caused a sharp fall in blood pressure and a rise in the pulse pressure A second and a third dose of venom injected after short intervals gave rise to serious respiratory disturbances deep, uneven breathing as well as disturbances in cardiac rhythm and extrasystole (fig 1)

The effect of the venom on striated muscle and on nerve conduction was studied in eight cats weighing 2 5 to 3 3 kg They were anaesthetized as previously mentioned and prepared for recording the contractions of the *tibialis anterior* and *gastrocnemius soleus* muscles in the right leg (BURN *et al* 1950, EDERY *et al* 1972) After the injection of 0 6–1 75 mg venom (diluted in 0 1–0 3 ml saline) the indirectly induced twitches of both muscles tested were gradually reduced, the *tibialis anterior* being in all cases more afffected than the *gastrocnemius soleus* The direct induced stimulus showed no changes in the muscle twitches (fig 2) thus indicating that the venom affects the neuromuscular junction but not the muscle cell membrane When

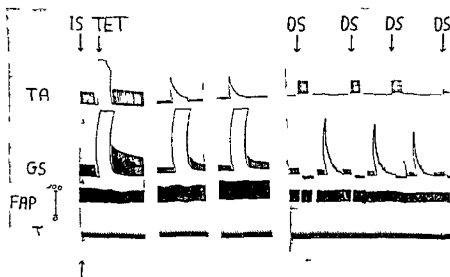


Fig 2 Reduction of indirectly induced twitches of striated muscle following injection of venom Injection (arrow) of 1 75 mg of dialysed venom sac extract into the femoral artery The amplitude of the indirectly (IS) induced contractions of *tibialis anterior* (TA) and *gastrocnemius soleus* (GS) was gradually reduced Direct stimulus (DS) of TA showed no changes in the muscle twitches

TET = tetanus

evoking a stimulus causing tetanus and injecting additional doses of venom the *tibialis anterior* was the first to reject the tetanus

Venom solution was injected into chicks weighing 34 g via the jugular vein 1–5 min after injecting 0 25–0 7 mg venom diluted by 0 1 ml saline, the following symptoms were observed dyspnoea, akinesia eyeclosure, brooding position, vertigo, flaccid paralysis and death

EDERY *et al* 1972 have described the activities of *Vespa orientalis* venom which by and large resemble those of *Paravespula germanica* but *V orientalis* venom produced paralysis of both directly and indirectly induced stimuli while the *P germanica* venom affected the indirect induced stimuli only The main substances in the *P germanica* are apparently large molecules and of a protein type that does not pass through the dialysis sac and is inactivated by boiling Apart from this protein like substance the venom contains a mixture of small molecular pharmacologically active substances which are probably responsible for the immediate changes in the cardio vascular system and stimulate contractions of smooth muscles Some of these activities are abolished by antagonists of acetylcholine (atropine) of 5 hydroxytryptamine (deseril®) and of histamine (mepyramine) JAQUES & SCHACHTER (1954) and PRADO *et al* (1966) have described the finding of biogenic amines and kinins in wasp venoms but apparently no work has been done on the characterization of the large molecular substances present in the venom which represent, as has been stated previously, the principal toxic component

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Different Properties of Microsomal UDP-Glucuronyltransferase in Buffalo Rat Liver and a Clonal Strain of Rat Hepatoma Cells Derived from the Same Rat Strain

By

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Abstract Optimal conditions for the synthesis of o aminophenol and p nitrophenol glucuronides by a clonal strain of rat hepatoma cells (MH_1C_1) in culture were established Properties of glucuronyltransferase (UDP glucuronate glucu ronyltransferase (acceptor unspecific), E C 2 4 1 17) in homogenates of cultured hepatoma cells, subcutaneous tumours derived from these cells in rats, as well as livers of Buffalo rats were studied In rat liver 83–90 % and 92–95 % of the glucuronyltransferase activity in homogenates to p nitrophenol and o aminophenol respectively were latent, the latency being most pronounced in male animals With homogenates of cultured hepatoma cells, on the other hand digitonin activated 1 3 and 1 8 fold only with p nitrophenol and o aminophenol as acceptors, other potential activators (Triton X 100, UDP N acetylglucosamine and diethylnitrosamine) were either without effect or inhibited the enzyme A 1 5–2 fold higher degree of activation of glucuronyltransferase was found in homogenates of hepatoma tumours derived from the same cells injected sub cutaneously into Buffalo rats The specific activity of fully activated glucuronyltransferase in homogenates of cultured hepatoma cells was however, 2–6 5 fold higher than that of the fully activated rat liver enzyme The rate of p nitrophenol glucuronide synthesis by cultures of hepatoma cells increased up to a concentration of 0 10 mM of the aglycone in the growth medium At concentra tions of 0 3 mM and higher, a peculiar lag period was seen before any glu curonide appeared in the medium This phenomenon was not seen with o amino phenol as acceptor and not in broken cell preparations with either substrate The maximal rate of o aminophenol glucuronidation in cultures of the hepatoma cells corresponded to that found for homogenates with 0 25–0 50 mM UDP glucuronate added to the incubation mixture This value is in good agreement with the presumed intracellular levels of UDP glucuronate in the liver cell *in vivo*

Key words UDP glucuronyltransferase – microsomes – rat liver – rat hepatoma cells

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The liver microsomal enzyme(s) glucuronyltransferase (UDP-glucuronate glucuronyltransferase (acceptor unspecific), E C 2 4 1 17) catalyse(s) the formation of glucuronides of a wide variety of biologically-important compounds of both endogenous and exogenous origin (DUTTON 1966, 1971) The activity *in vitro* of this enzyme is increased several-fold by many compounds Thus detergents (LUEDERS & KUFF 1967, WINSNES 1969) UDP-*N*-acetylglucosamine (POGELL & LELOIR 1961, WINSNES 1969) and diethylnitrosamine (STEVENSON *et al* 1968, WINSNES 1969) when added to liver homogenates may enhance glucuronyltransferase activity up to 20-fold The available evidence suggests that this is an activation at V_{max} (VESSEY & ZAKIM 1971, WINSNES 1972) indicating that more active sites are made accessible for the substrate(s) Whether this activation is due to effects on the enzyme protein itself or on the microsomal membrane environment is open to speculation It is also not known whether this activation *in vitro* reflects regulatory properties of physiological significance

For better evaluation of the significance of glucuronyltransferase activity measurements performed in homogenates (with and without activators) in comparison with the rate of glucuronide synthesis in intact cells is highly desirable However, this approach has hitherto been hampered by the lack of a line of cells in which the synthesis of glucuronides remains intact We have used a clonal strain of rat hepatoma cells (MH_1C_1) established in culture from the transplanted Morris hepatoma number 7795 by RICHARDSON *et al* (1969) These cells perform a series of liver-specific functions after serial propagation *in vitro* for years, they produce serum albumin, tyrosine aminotransferase, the ninth component of complement (TASHJIAN *et al* 1970), blood clotting factors (RUGSTAD *et al* 1972) and several other rat plasma proteins (GAUDERNACK *et al* 1973) These cells have previously been found to glucuronidate bilirubin (RUGSTAD *et al* 1970) and *p* aminophenol (DYBING & RUGSTAD 1972) The hepatoma cells might therefore offer a good model for studies on the regulation of glucuronide synthesis

Materials and Methods

Chemicals of analytical grade were obtained from the same sources as described earlier (WINSNES 1969)

Glucuronide determinations *o*-Aminophenol glucuronide was measured as described by LEVVY & STOREY (1949) Reduction in absorbance at 400 nm in the case of *p* nitro phenol (ISSELBACHER 1956) and reduction in fluorescence at 460 nm in the case of 4-methylumbelliferone (ARIAS 1962) were used as measure of the amount of glucuronide formed with these acceptor substrates

Methods of cell culture A clonal strain of rat hepatoma cells derived from the transplantable Morris rat hepatoma No 7795 (RICHARDSON *et al* 1969) which originated

from a Buffalo rat, was grown in Falcon plastic tissue culture flasks (250 ml) in Dulbecco's modification of Eagle's medium supplemented with 15 % horse serum and 2.5 % foetal calf serum and antibiotics as described by RUGSTAD *et al* (1970)

Glucuronide synthesis by intact hepatoma cells was measured by incubating the cells at 37° with varying concentrations of *o*-aminophenol, *p* nitrophenol and 4 methylumbelliferone as substrates At the beginning of each experiment the growth medium was removed and the cells washed once with fresh prewarmed medium without added serum A prewarmed mixture of 9.5 ml Dulbecco's medium without serum and 0.5 ml stock solution of substrate was then added This mixture had been bubbled through with 5 % CO_2 in air before the incubation started Ascorbic acid was used as anti oxidant for *o* aminophenol and was present in 1.0 mM final concentration in the medium Ethanol was used to dissolve *p* nitrophenol giving a final concentration of approximately 0.05 % (v/v) ethanol in the medium Bovine albumin was used as carrier for 4 methylumbelliferone in a concentration of 20 mg per 1.0 mM of substrate, thus probably giving a constant percentage of free substrate at varying absolute concentrations At desired time intervals 0.5 ml aliquots were removed for measurement of *o* aminophenol glucuronide, *p* nitrophenol and 4-methylumbelliferone

Homogenate experiments Glucuronyltransferase was studied in homogenates of 1) cultured hepatoma cells, 2) subcutaneous tumours from Buffalo rats (100–200 g weight) injected with a hepatoma cell suspension 4–5 weeks before sacrifize and 3) livers from Buffalo rats The homogenates were prepared in isotonic KCl solution with a homogenizer (submerged in ice water) with Teflon pestle at high speed for 1–2 min The homogenate was spun at 1000 × g for 5 min and the supernatant was used for enzyme assay Homogenates of hepatoma cells were also prepared with a micro modification of the Potter Elvehjem glass to glass homogenizer (volume about 0.5 ml) Grinding was performed at low speed for 10 min No significant difference in specific activity or degree of activation by digitonin was noted between cell homogenates prepared with Teflon or glass pestle Ultrasonication was avoided as homogenization technique since this treatment activates glucuronyltransferase (HENDERSON 1970) The cell homogenates were usually prepared from 10 culture flasks (a total of about 50 mg protein) The cells were harvested by incubation and subsequent shaking with 0.02 % EDTA in phosphate buffered saline The cells were centrifuged down and washed once in isotonic saline

Glucuronyltransferase activity towards *o*-aminophenol and *p* nitrophenol in homogenates was measured in 0.5 ml final volume with Tris maleate buffer at pH 7.4 10 mM $MgCl_2$ was present with *o* aminophenol as acceptor With this substrate the amount of protein in the incubation mixture was 0.15–0.40 mg in the case of hepatoma tissue whereas with rat liver 2–5 mg protein was used in the assay The incubation time was usually 10–15 min, but at low protein concentration or with non activated enzyme incubations were often extended to 30 min The linearity with time and increasing protein concentration was satisfactory if not more than 25 % of the acceptor substrate was consumed during incubation In order to ensure such linearity, pilot incubations were necessary with each batch of hepatoma homogenate to determine the suitable incubation time and protein concentration With *p* nitrophenol as acceptor both the protein concentration and the incubation time had to be reduced to one half or one third of that mentioned above for *o* aminophenol as acceptor If not otherwise stated the substrate concentrations were 0.5 mM for aglycone and 2 mM for UDP glucuronate Maximal activation was obtained with the following concentrations of the activators, 2 mM UDP *N* acetylglucosamine and 15 mM diethylnitrosamine, whereas with digitonin 0.10 % (w/v) was optimal in the case of liver homogenates and

0.05 % in homogenates of cultured hepatoma cells. Further details on the enzyme assay methods are given elsewhere (WINSNES 1969).

Protein concentrations were determined by OYAMA & EAGLE's (1956) modification of Lowry's method with bovine serum albumin as standard.

The effect of orotic acid on o-aminophenol glucuronidation in intact hepatoma cells was tested with 0.1, 0.5 and 1.0 mM orotic acid added in the culture medium together with the aglycone. Two cell culture flasks were also pre-incubated for one hour with 0.5 mM orotic acid before addition of o-aminophenol.

Histological examination of subcutaneous tumour tissue was performed after fixation in 4 % formaldehyde and staining with haematoxylin-eosine, Van Gieson's connective tissue stain and Gomori's reticulum stain.

Results

Glucuronide synthesis in intact hepatoma cells

The rate of appearance of *o*-aminophenol glucuronide in the culture medium during incubation of hepatoma cells at 37° is shown in fig. 1. At concentrations of *o*-aminophenol up to 0.2 mM, the rate of glucuronide appearance diminished with time probably because of depletion of aglycone. With 0.50 mM *o*-aminophenol in the medium the reaction was linear for five hours. The maximal rate of glucuronide synthesis with *o*-aminophenol as acceptor was obtained at about 0.5 mM of substrate, and the rate was constant up to 1.0 mM substrate concentration.

At low concentrations (0.025–0.10 mM) of *p*-nitrophenol (fig. 1) and 4-methylumbelliferone (not shown in the figure) a constant rate of substrate consumption was found for approximately one hour. At higher concentrations of *p*-nitrophenol and 4-methylumbelliferone a lag period appeared and increased in length with increasing substrate concentrations. Thus with 0.5 mM *p*-nitrophenol in the medium no significant consumption of substrate was found during the first 2 hours. After that time the reaction rate increased gradually up to 4 hours. With 0.30 mM *p*-nitrophenol only a very short lag period was found, and the reaction rate was constant from 40 min to 4 hours. Similar results were obtained with 4-methylumbelliferone as substrate.

The maximal rates of conjugation per mg protein and per hour with intact cells were about 42 nmol at 0.5 mM *o*-aminophenol (fig. 1), 58 nmol at 0.10 mM *p*-nitrophenol (fig. 1) and 105 nmol at 0.10 mM 4-methylumbelliferone.

One set of experiments with 0.5 mM *o*-aminophenol and varying concentrations (0, 0.5, 1.0, 2.0 mM) of UDP-glucuronate in the medium was performed. The amount of *o*-aminophenol glucuronide synthesized during the first two hours was exactly the same, indicating that UDP-glucuronate was unable to pass through the membrane barriers.

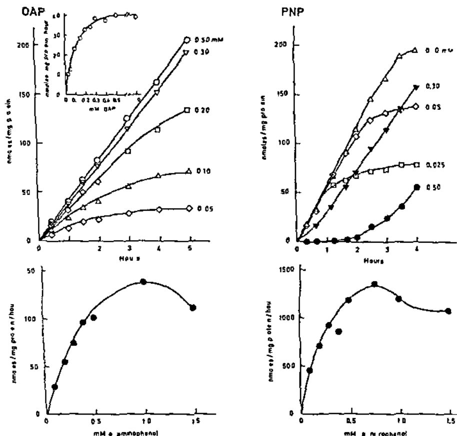


Fig 1 o Aminophenol and p nitrophenol glucuronide synthesis in intact and broken hepatoma cells at varying aglycone concentrations Intact cells in culture (upper graphs) and hepatoma cell homogenate (lower graphs) were incubated with varying concentrations of o aminophenol (left graphs) and p nitrophenol (right graphs) as indicated in the figures Other conditions were as described in Materials and Methods

Orotic acid is a precursor of uridine nucleotides, and a beneficial effect of this acid on the hyperbilirubinaemia of prematurity has been reported (KINTZEL *et al* 1971) However no effect of orotic acid (in the concentrations tested) on *o*-aminophenol glucuronidation rates was seen during four hours incubation

UDP-glucuronyltransferase activities in homogenates of cultured hepatoma cells

With *o*-aminophenol as acceptor the maximal activity was reached at 1 0 mM whereas 1 5 mM of *o* aminophenol was slightly inhibitory (fig 1)

With intact cells a similar curve was obtained although there was no significant activity increase in the *o*-aminophenol concentration range 0 3–1 0 mM Thus the concentration (0 5 mM) of acceptor substrate used routinely in the enzyme assay limited the glucuronidation rate in homogenates to about 70 % of the maximal, but to increase its concentration further would obscure comparison with glucuronidation rate in intact cells

The maximal activity towards *p* nitrophenol was reached at 0 75 mM of acceptor (fig 1) which is considerably higher than in intact cells where the maximal rate was reached at 0 10 mM The peculiar lag period seen in intact cells at 0 30 and 0 50 mM concentration of *p* nitrophenol was not seen with homogenates

The pH optimum of 'native" *o*-aminophenol glucuronyltransferase in homogenates of cultured hepatoma cells was 7 8, whereas with *p* nitrophenol as substrate the optimum was at pH 6 2 (fig 2), both values being similar to that reported for the corresponding detergent activated mouse and rat liver enzymes (WINSNES 1969, HOWLAND *et al* 1971) pH 7 4 was, however, used routinely in homogenate experiments with both acceptors to make comparison with intact cell glucuronidation easier, as pH in the growth medium was kept at 7 4

The *o* aminophenol glucuronyltransferase activity increased considerably in the UDP-glucuronate concentration range 0 125–8 0 mM and had not reached its maximum at 8 mM (fig 3) The Lineweaver-Burk plot was linear and the apparent $K_{UDP\ glucuronate}$ value was 2 7 mM (1 7, 3 1 and 3 3 in different experiments) while the apparent V_{max} was 219 nmol/mg protein per hour (200, 167 and 290 in different experiments) The enzyme activity found in intact cells (42 nmol/mg protein per hour) was reached at

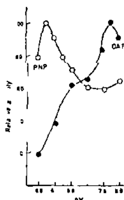


Fig 2 pH-optimum of "native" *o*-aminophenol and *p* nitrophenol glucuronyltransferase in homogenates of cultured hepatoma cells

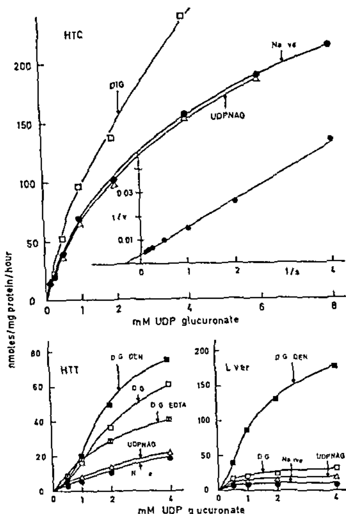


Fig 3 *o* Aminophenol glucuronide synthesis in homogenates of cultured hepatoma cells (HTC) hepatoma tumour (HTT) and Buffalo rat liver (Liver) at varying UDP glucuronate concentrations and f xed aglycone concentration (0 5 mM) Assays were performed with native enzyme as well as w th optimal concentrations of d gitonin (DIG) UDP *N* acetylglucosam ne (UDPNAG) diethylnitrosamine (DEN) and EDTA added

a UDP glucuronate concentration between 0 25 and 0 50 mM which is similar to the concentrations reported for this nucleotide sugar in rat liver, 0 28 μmol per g wet tissue (KEPPLER *et al* 1970), 0 12 μmol per g wet tissue (ZHIVKOV 1970)

Although the UDP-glucuronate saturation curve of *p*-nitrophenol glucuronyltransferase appeared to be a hyperbola, the more sensitive double reciprocal plot revealed an abrupt transition at 1 mM UDP glucuronate (fig 4) This phenomenon has recently also been described for some forms of the mouse and rat liver enzyme (WINSNES 1972) It may (among other possibilities) indicate the existence of two different enzymes or two active sites with different affinity on one enzyme (CONWAY & KOSHLAND 1968, LEVITZKI & KOSHLAND 1969) The apparent $K_{UDP\ glucuronate}$ values were 0 6 and 1 5 mM respectively The apparent V_{max} values were 670 and 1080 nmol per mg protein per hour which is 10–15 times higher than the maximal glucuronidation rate obtained with intact cells in culture However, since glucuronide synthesis with intact cells was inhibited at 0 5 mM of *p*-nitrophenol (used in the incubation mixtures with homogenates) no adequate comparison with the results obtained in homogenates can be performed

Since rat liver glucuronyltransferase activity *in vitro* is enhanced markedly by detergents, UDP-*N*-acetylglucosamine and, with *o*-aminophenol as substrate, also by diethylnitrosamine (STEVENSON *et al* 1968, WINSNES 1969), it was of interest to test the effects of these agents on the hepatoma cell enzyme As seen from tables 1 & 2, the activity could be increased 1 8 and 1 3-fold by digitonin with *o* aminophenol and *p*-nitrophenol as acceptors Neither UDP-*N*-acetylglucosamine nor diethylnitrosamine (with *o*-aminophenol as acceptor only) activated glucuronyltransferase in homogenates of these cells in contrast to the enzyme in rat liver

UDP-glucuronyltransferase activities in homogenates from hepatoma tumour and Buffalo rat liver

Since culture conditions might possibly be responsible for the atypical activation characteristics of glucuronyltransferase in hepatoma cell homogenates a suspension of these cells was injected subcutaneously in 2–3 weeks-old Buffalo rats of either sex After 4–5 weeks a visible tumour usually developed These were excised when weighing about 1 gram and homogenates of the tumour as well as of the host liver prepared

Figs 3 and 4 and tables 1 and 2 show that a slightly higher degree of activation by digitonin was found for glucuronyltransferase in tumour homogenates as compared with homogenates of cultured cells Diethylnitrosamine and UDP-*N* acetylglucosamine caused no activation of the transferase in cell culture homogenates, whereas in tumour homogenates a 10 respectively 40 % increase in activity was caused by these agents when *o*-aminophenol was used as acceptor Diethylnitrosamine, however, enhanced the enzyme activity 2 6 (male) – 4 6 (female) times, and UDP-*N*-acetylglucosamine about 4-fold when added to rat liver homogenates (tables 1 and 2)

Table 1

o Aminophenol glucuronyltransferase activities (at 2 mM UDP glucuronate) in homogenates of cultured hepatoma cells, hepatoma tumour and Buffalo rat liver Activities (single values or mean ± S E M of *n* number of experiments) are given as nmol per mg protein per hour Relative activities are given in brackets Other conditions were as described in Materials and Methods

Additions to standard assay mixture	Cultured hepatoma cells	Hepatoma tumour female host	Hepatoma tumour, male host	Female Buffalo rat	Male Buffalo rat
None	98 ± 1 0 (1 0) n = 10	10 1 – 15 6 (1 0) n = 2	17 2 (1 0) n = 1	3 4 – 4 1 (1 0) n = 2	4 6 ± 0 4 (1 0) n = 5
UDP *N* acetylglucosamine	100 ± 4 9 (1 0) n = 3	15 9 – 19 9 (1 4) n = 2	---	---	17 3 ± 0 9 (3 8) n = 3
Digitonin	178 ± 5 7 (1 8) n = 7	41 2 ± 2 0 (3 2) n = 3	---	11 6 – 11 6 (3 1) n = 2	30 4 – 37 2 (7 4) n = 2
Digitonin + diethylnitrosamine	128 (1 3) n = 1	45 3 ± 3 2 (3 5) n = 4	45 0 (2 6) n = 1	50 0 (13 3) n = 1	89 0 ± 8 9 (19 5) n = 3

Table 2

p Nitrophenol glucuronyltransferase activities (at 2 mM UDP glucuronate) in homogenates of cultured hepatoma cells hepatoma tumour and Buffalo rat liver Activities (single values or mean ± S E M of *n* number of experiments) are given as nmol per mg protein per hour Relative activities are given in brackets Other conditions were as described in Materials and Methods

Additions to standard assay mixture	Cultured hepa toma cells	Hepatoma tumour, female host	Hepatoma tumour, male host	Female Buffalo rat	Male Buffalo rat
None	756 ± 119 (1 0) n = 3	374 (1 0) n = 1	(202–295) (1 0) n = 2	25 2 ± 2 8 (1 0) n = 3	25 2 ± 0 9 (1 0) n = 5
UDP *N* acetylglucosamine	830 (1 1) n = 1	378 (1 0) n = 1	266 (1 1) n = 1	---	108 0 ± 1 7 (4 3) n = 4
Digitonin	975 ± 19 5 (1 3) n = 6	584 (1 6) n = 1	(435–480) (1 8) n = 2	149 8 (5 9) n = 1	252 0 ± 13 8 (10 0) n = 4

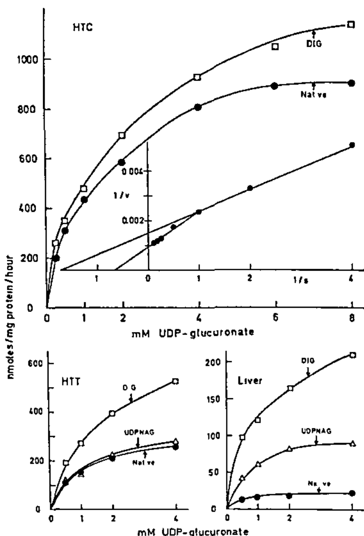


Fig 4 *p* Nitrophenol glucuronide synthesis in homogenates of cultured hepatoma cells (HTC) hepatoma tumour (HTT) and Buffalo rat liver (Liver) at varying UDP glucuronate concentrations and fixed aglycone concentration (0 5 mM) Assays were performed with "native" enzyme as well as with optimal concentrations of digitonin (DIG) and UDP *N* acetylglucosamine (UDPNAG)

Although the "native" *o*-aminophenol glucuronyltransferase activity in hepatoma tumour was 3 4–3 7 times higher than in the host livers, the maximally activated enzyme (both digitonin and diethylnitrosamine present) was more active in liver (table 1) This is in contrast to the finding for *p* nitrophenol glucuronyltransferase where activities (both "native" and ac-

tivated enzyme) in liver always were considerably lower than in tumour With both *o*-aminophenol and *p* nitrophenol as acceptors the specific activities in homogenates of hepatoma tumour were ¼–½ of that in homogenates of cultured hepatoma cells (tables 1 and 2) No significant conjugation of *o*-aminophenol was detected in homogenates of subcutaneous tissue from these rats

Both with tumour and liver homogenates atypical, slightly sigmoid UDP-glucuronate saturation curves for *o*-aminophenol glucuronyltransferase were found (fig 3) The sigmoid form was not seen when EDTA was added to 10 mM concentration, possibly because of inhibition of UDP-glucuronate pyrophosphatase activity which otherwise would result in artifically low apparent glucuronyltransferase activities at low substrate levels (POGELL & LELOIR 1961) With homogenates of cultured hepatoma cells (*o*-aminophenol) and for *p* nitrophenol as substrate with all enzyme sources no sigmoid part of the curve seemed to be present, possibly because less protein, and consequently less pyrophosphatase, was present in the incubation mixtures Apparent $K_{UDP\ glucuronate}$ and V_{max} values could therefore not be calculated from the present experiments with tumour and liver-homogenates (without EDTA added) Comparison of the specific activities at 2 mM UDP-glucuronate (tables 1 and 2), however, probably give a fair idea of the relative amounts of enzyme in the different tissues Hepatoma cells in culture undoubtably have a considerably higher specific activity of both *p* nitrophenol and *o*-aminophenol glucuronyltransferase than rat liver from the host animals (tables 1 and 2)

Discussion

It is evident from the present report that in "native" homogenates of cultured hepatoma cells 56–77 % of the maximal glucuronyltransferase activity (digitonin activated preparations) is measured In contrast the degree of latency of this enzyme in rat liver is considerable, only 5–8 % (*o*-aminophenol as acceptor) respectively 10–17 % (*p*-nitrophenol as acceptor) of the activity of optimally activated enzyme (at 2 mM UDP glucuronate) is measurable in "native" preparations (tables 1 and 2)

There are mainly two possible explanations of the different degree of latency found in hepatoma cells as compared with liver The endoplasmic reticulum membranes of the hepatoma cells might also be permeable to charged substances, such as UDP glucuronate, to a greater extent than the membranes found in rat liver Uncharged substances such as *o*-aminophenol and *p* nitrophenol have probably free access to microsomal enzymes (NILSSON *et al* 1971) It is known that fast growing hepatomas may show a

simplification of the endoplasmic reticulum (HRUBAN *et al* 1972) It is also possible that nutritional factors can affect the degree of latency of glucuronyltransferase Thus the latency increased slightly when the cells were transplanted subcutaneously In this connection it is interesting to note that severe protein deficiency in rats was associated with increased glucuronyltransferase activity, whereas the activity of several other drug-metabolizing enzymes decreased considerably (WOOD & WOODCOCK 1970, WOODCOCK & WOOD 1971) Since "native" glucuronyltransferase activity only was measured in these studies, this stimulation by protein deficiency might have been caused by a decreased latency of the enzyme

Another possible explanation for the different degrees of latency is the existence of different conformational forms of the enzyme protein itself Diethylnitrosamine probably exerts its primary effect on the enzyme and not the microsomal membrane because 1) the effect is confined to one species (rat) and two substrates (*o*-aminophenol and paracetamol) and 2) it activates the enzyme "solubilized" by digitonin (STEVENSON *et al* 1968) Since there is no activating effect of diethylnitrosamine on the hepatoma cell enzyme, this might therefore indicate that a different enzyme form is present in these cells In this connection it is of interest that the enzyme from newborn rats cannot be activated by this agent (WINSNES 1971a) Possibly therefore the hepatoma cells contain an ontogenically early type of glucuronyltransferase This would agree with the frequent finding that enzyme forms characteristic for the embryonic period tend to dominate in malignant cells (SCHAPIRA & HATZFELD 1972)

In cultured hepatoma cell and tumour homogenates no or only very slight activation by UDP-*N*-acetylglucosamine was found whereas digitonin activated 1 3–3-fold This dissociation between the effect of detergent and the nucleotide-sugar in its activation effects is similar to that seen in homogenates treated with thiol blocking agents (WINSNES 1971c) Conformational change in both the endoplasmic reticulum membranes as well as the enzyme itself could however, explain such differences between the hepatoma and liver tissue enzyme

Recently it was shown that apparent $K_{UDP\ glucuronate}$ values of 'native' glucuronyltransferase were considerably lower than those of the activated enzyme (WINSNES 1972) The apparent $K_{UDP\ glucuronate}$ values of 'native" hepatoma cell glucuronyltransferase are of the high type i e similar to those of the activated form of liver enzyme The pH optimum of *p* nitrophenol glucuronyltransferase was also that characteristic for the activated form of enzyme (fig 2, WINSNES 1969)

The decreased activity in preparations from the subcutaneous tumour preparations as compared with homogenates from cultured cells cannot be explained by the admixture of cells of other types e g fibroblasts in the

tivated enzyme) in liver always were considerably lower than in tumour With both *o*-aminophenol and *p*-nitrophenol as acceptors the specific activities in homogenates of hepatoma tumour were ¼–½ of that in homogenates of cultured hepatoma cells (tables 1 and 2) No significant conjugation of *o*-aminophenol was detected in homogenates of subcutaneous tissue from these rats

Both with tumour and liver homogenates atypical, slightly sigmoid UDP-glucuronate saturation curves for *o*-aminophenol glucuronyltransferase were found (fig 3) The sigmoid form was not seen when EDTA was added to 10 mM concentration, possibly because of inhibition of UDP-glucuronate pyrophosphatase activity which otherwise would result in artifically low apparent glucuronyltransferase activities at low substrate levels (POGELL & LELOIR 1961) With homogenates of cultured hepatoma cells (*o* aminophenol) and for *p* nitrophenol as substrate with all enzyme sources no sigmoid part of the curve seemed to be present, possibly because less protein, and consequently less pyrophosphatase, was present in the incubation mixtures Apparent $K_{UDP\ glucuronate}$ and V_{max} values could therefore not be calculated from the present experiments with tumour and liver-homogenates (without EDTA added) Comparison of the specific activities at 2 mM UDP-glucuronate (tables 1 and 2), however, probably give a fair idea of the relative amounts of enzyme in the different tissues Hepatoma cells in culture undoubtably have a considerably higher specific activity of both *p* nitrophenol and *o*-aminophenol glucuronyltransferase than rat liver from the host animals (tables 1 and 2)

Discussion

It is evident from the present report that in "native" homogenates of cultured hepatoma cells 56–77 % of the maximal glucuronyltransferase activity (digitonin-activated preparations) is measured In contrast the degree of latency of this enzyme in rat liver is considerable, only 5–8 % (*o*-aminophenol as acceptor) respectively 10–17 % (*p*-nitrophenol as acceptor) of the activity of optimally activated enzyme (at 2 mM UDP-glucuronate) is measurable in "native" preparations (tables 1 and 2)

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Alterations in Neurochemical and Behavioural Parameters in the Mouse Induced by Low Doses of Methyl Mercury

By

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Abstract The effects on mouse behaviour in the open field situation of controlled single doses of methyl mercury (MeHg) (1, 5, 10 mg Hg/kg) were investigated at varying times after intraperitoneal injection (1, 3, 72 hours) In an effort to correlate behavioural and biochemical data, the effects of dose and time after dose on the levels of selected glycolytic pathway intermediates, α-glycerophosphate, adenine nucleotides and phosphocreatine were monitored A very good correlation between brain biochemistry and behavioural effects of MeHg was observed That is the dose response relationship for the open field task correlated with alterations in levels of metabolic intermediates At 1 and 3 hours after administration of MeHg, when the levels of the metabolic intermediates were significantly different from those of controls, altered behaviour was observed At 72 hours post administration, when the biological parameters were approaching control values, a return to normal behaviour was observed

Key words Methyl mercury – neurochemistry – behaviour – mice

There has been extensive investigation of the tissue distribution, pathology and neurology of overtly toxic concentrations of methyl mercury (MeHg) in a variety of species (See ex *Methyl Mercury in Fish*, 1971) It has been observed that the distribution of MeHg in animals is characteristically different from that of inorganic mercury (Berlin & Ullberg 1963a, b, c) Neurological symptoms dominate the picture in cases of accidental and experimental MeHg intoxication A latent period is often observed in humans and other mammals from the time of exposure to the onset of overt

neurological symptoms In mice, the dominant symptoms are cerebellar dysfunction and a characteristic hyper reflexia (SAITO *et al* 1961, SUZUKI 1969) On the cellular level, damage is first observed to occur to neuroglia and granule cells Purkinje cells appear unaffected until the later stages of poisoning (TAKEUCHI 1968a, b, 1970) Much the same pattern of destruction is observed in myelinating cultures of mouse cerebellum (KIM 1971) along with a secondary degeneration of myelin sheaths

In contrast, little is known of the effects of low doses of MeHg (i e, doses which do not induce classical symptoms of MeHg intoxication) either at the cellular molecular level (BERLIN *et al* 1965, CHANG & HARTMAN 1972a, b, CREMER 1967, KUWAHARA 1970, MIYAKAWA *et al* 1969, 1970, PATTERSON & USHER 1971, YOSHINO *et al* 1966a, b) or on behaviour (BROWN *et al* 1972, EVANS & KOSTYNIAK 1972, HUGHES *et al* 1972, POST *et al* 1972, SPYKER *et al* 1972) In the behavioural studies using MeHg, generally, only the effects of relatively large doses have been investigated and/or the amount of MeHg uptake was not controlled Thus, EVANS & KOSTYNIAK (1972) produced gross intoxication in pigeons while the rats of POST *et al* (1972) may have received up to 20 mg Hg/kg depending upon the amount of MgHg that passed through the digestive tract and was excreted The quantity of MeHg taken-up by the mice in BROWN *et al's* (1972) experiments, was certainly uncontrolled

The lack of systematic knowledge of the effects of low doses of MeHg on neurochemistry and behaviour cannot be over emphasized Given the deteriorating environmental situation, the acquisition of such knowledge must become of increasing concern Thus, utilizing the mouse as a model system, we have undertaken an investigation of the time dependent effects *of controlled, low doses (sub LD 50) of MeHg on* (a) *behaviour in* the open field situation, and (b) the levels of adenine nucleotides, phosphocreatine, α glycerophosphate and the activity of the glycolytic pathway in the cerebellum and cerebral cortex

The glycolytic pathway was chosen because of its central importance in energy production in the brain (LAJTHA 1970) Mice were selected because they have been used in many psychopharmacological studies (CANDLAND & NAGY 1969 LABARBA & HODGE 1970, MCREYNOLDS *et al* 1967, NAGY & FORREST 1970, NAGY & GLASER 1970, NAGY & HOLM 1970) Since the open field was first used in psychopharmacological studies (JANSSEN *et al* 1960) different drugs have been shown to affect various measures of the behaviour of mice (and rats) in the open field (AHTEE & KARKI 1968, BRIMBLECOMBE 1963, BROADHURST *et al* 1959, ERIKSSON & WALLGREN 1967, JANSSEN *et al* 1960, MORRISON & LEE 1968, RYALL 1958) Thus, it seemed reasonable to use the open field as a first step in evaluating some of the effects of MeHg on the behaviour of mice

Materials and Methods

I Biochemical studies

Reagents All analytical enzymes were purchased from Boehringer (Mannheim Corp, New York, N Y) except rabbit muscle myokinase which was obtained from Sigma (Chemical Co, St Louis, Mo) All auxiliary substrates and co-factors were obtained from Sigma ^{203}Hg labelled methyl mercury chloride was obtained from New England Nuclear (Boston, Mass) (> 99 % radiochemical purity) Stable methyl mercury hydroxide (MeHgOH) was purchased from Ventron (Beverly, Mass) All other chemicals used were of reagent or fluorescent grade

Preparation of animals and brain samples Adult male Swiss-Webster mice (Carworth Breeding Laboratories, New York, N Y) weighing 25–32 g were used For measurements of brain metabolic intermediates, the animals were decapitated and placed directly into Freon 12 (CCl_2F_2) maintained at −150° with liquid N_2 The heads were stored at −80° before preparation for analysis

Dosing of animals

A Animals used for measurement of levels of metabolic intermediates and behaviour in the open field task

A stock solution of MeHgOH containing 10 mg Hg/ml was prepared in 0 14 M NaCl Thin layer chromatography on cellulose and silica gel G in 3 M NH_4OH showed complete conversion of the OH to the Cl salt Dilutions containing 0 1, 0 5 or 1 0 mg Hg/ml were administered intraperitoneally in aliquots of 1 ml per 100 g body weight (b w) corresponding to doses of 1, 5 and 10 mg Hg/kg respectively Control animals were injected with 0 14 M NaCl (1 ml/100 g b w)

The analyses for levels of intermediates were performed on groups of 12 animals i.e 6 experimentals and 6 controls at each dose and time studied Animals were sacrificed at 1 and 3 hours after each administration of the three dose levels and at 1, 3 and 72 hours after the highest dose

B Animals used for time dependent distribution of ^{203}Hg labelled MeHg

A solution of 1 mg Hg/ml MeHgOH (containing 9 72 µci/ml of ^{203}Hg labelled methyl mercury chloride) was prepared in 0 14 M NaCl and administered intraperitoneally to twenty animals (1 ml/100 g b w) This corresponded to 10 mg Hg/kg and approximately 2 µci per animal

Animals were sacrificed by cervical dislocation in groups of 4 at 1, 3, 24 and 72 hours post administration Two animals were sacrificed at 7 days One animal died at 48 hours and another at 5 days post administration

Preparation of tissue and analytical methods

A For levels of intermediates

Tissue was prepared essentially as described by Kauffman *et al* 1969 Analyses of levels of metabolic intermediates were performed using slightly modified standard fluorometric techniques (Lowry *et al* 1964, Maitra & Estabrook 1964) to measure the appearance of NADH/NADPH or disappearance of NADH (NADH = reduced nicotinamide adenine dinucleotide, NADPH = reduced nicotinamide adenine dinucleotide phosphate The key to all other abbreviations used is found in table 1)

B For time dependent distribution of ^{203}Hg

Whole blood (approximately 100 μl) was obtained from the heart and pericardial cavity immediately following sacrifice The brain was removed and dissected into the cerebellum and cerebral cortex All tissues were placed in tared polyethylene vials, weighed and counted in a Packard Model 3003 gamma scintillation spectrometer Mercury concentrations were determined by comparison with a standard prepared from the injection solution Counting efficiency averaged 35 %

C Statistical analysis

The data for levels of brain glycolytic intermediates α glycerophosphate, adenine nucleotides, and phosphocreatine were analyzed by analysis of variance The con current control mean was subtracted from the individual experimental values (μmol/g wet wt) to compensate for day to day variations in base line levels of intermediates An approximate 3 way analysis of variance was performed separately on the values for each intermediate measured using a 4 × 2 × 2 experimental design and the NYBMUL computer program (NYBMUL 1969) The factors in the design represented dose (0, 1, 5 or 10 mg Hg/kg) time (1 and 3 hours after dose) and brain region (cerebellum or cortex) *Data from the 10 mg Hg/kg dose was subjected to a one way analysis of* variance with regard to time (1, 3 and 72 hours) irrespective of region

Student s t test for paired data was used for analysis of the differential uptake of ^{203}Hg labelled MeHg by different brain regions The concentration of ^{203}Hg in the cerebellum and cortex of each animal at all times represented the pairs

II Behavioural studies

Subjects One hundred twenty 6 to 8 week old, male Swiss Webster mice were used (*Ss*)

Apparatus The open *field apparatus was a 3 feet by 3 feet area divided into* 36 six inch squares and enclosed by 24 inch high walls The field was white with black dividers and was uniformly illuminated from above with a 30 watt fluorescent lamp

Procedure The animals were dosed as in the biochemical studies Each of the 4 dose conditions was divided into 3 groups One of the groups was observed in the open field 1 hour after injection, one group 3 hours after injection and one group 72 hours after injection This procedure generates a 4 × 3 factorial design having 12 groups There were 10 animals in each group

Each mouse was placed in a particular square near the center of the open field and was observed for 10 minutes Ambulations were scored as the number of squares entered (all 4 feet) by the *S* during each minute of the observation period A rearing was recorded each time a *S* stood up on its hind legs The observers did not know what dosage group the *Ss* were in.

Results

Biochemical The values of control levels of the metabolic intermediates under investigation are recorded in table 1. Fig 1 illustrates the alterations in brain (cerebral cortex plus cerebellum) levels of six glycolytic inter-

Table 1.

Intermediate	Control level(a) of intermediates in cerebellum and cortex
Glucose 1 phosphate (G1P) (73)(b)	0 006 ± 0 0007
Glucose-6-phosphate (G6P) (81)	0 041 ± 0 0010
Fructose 6-phosphate (F6P) (77)	0 008 ± 0 0002
Fructose 1,6-diphosphate (FDP) (71)	0 143 ± 0 0099
Dihydroxyacetonephosphate (DHAP) (77)	0.38 ± 0 0009
α Glycerophosphate (α GOP) (71)	0.271 ± 0 0107
Pyruvate (Pyr) (77)	0 118 ± 0 0038
Phosphocreatine (PC) (57)	1 219 ± 0 0311
Adenosine triphosphate (ATP) (61)	1 452 ± 0 0347
Adenosine diphosphate (ADP) (64)	0 604 ± 0 0235
Adenosine monophosphate (AMP) (75)	0 359 ± 0 0149

(a) Levels expressed in μmol/g wet weight of brain ± S E M

(b) Number of samples analyzed

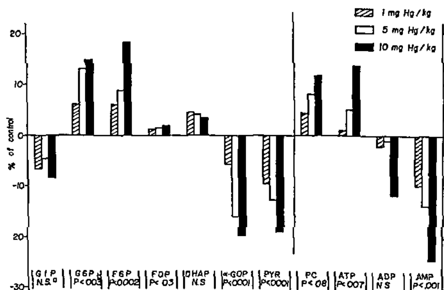


Fig 1 Changes in mouse brain (cerebral cortex plus cerebellum) levels of glycolytic intermediates, αGOP, PC, and adenine nucleotides induced by varying doses of MeHg (both 1 and 3 hours post intraperitoneal injection)

a Not significantly different from control

b Level of significance from 3 way analysis of variance

mediates, αGOP, PC, and adenine nucleotides induced by MeHg at 1 and 3 hours post administration

A dose related response is indicated for all significant increases and decreases in the levels of the intermediates investigated No significant interactions between the main effects of dose and time were observed

When data for the three dose levels at 1 and 3 hours post injection are analyzed in the 4 × 2 × 2 factorial design, no significant main effect of time is observed for any intermediate However, when data for the dose of 10 mg Hg/kg (at 1, 3 or 72 hrs) are analyzed separately with time, significant changes are found Fig 2 shows the percentage change from control values as a function of time for the glycolytic intermediates, αGOP, PC and adenine nucleotides In general, the pattern is one of greater changes from control levels at 1 and 3 hours than at 72 hours for glycolytic intermediates, ADP and AMP The changes in FDP, PYR and AMP are significant with regard to time in a one way analysis of variance ATP levels were essentially identical while those of PC increased slightly

MeHg induced changes in the levels of glycolytic intermediates, αGOP, adenine nucleotides and PC varied as a function of the brain region The

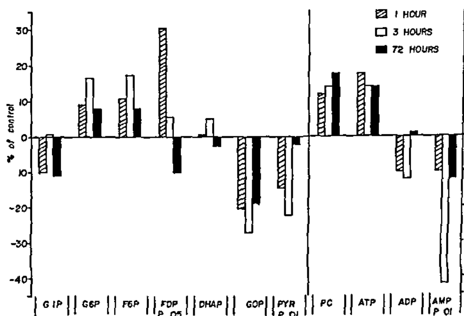


Fig 2 Changes in mouse brain (cerebral cortex plus cerebellum) levels of glycolytic intermediates, αGOP, PC, and adenine nucleotides induced by 10 mg Hg/kg MeHg at various times after intraperitoneal injection

[a] Level of significance from one way analysis of variance

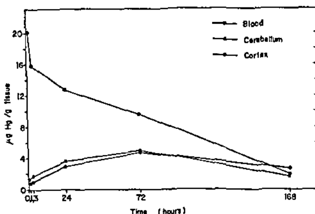


Fig 3 Time dependent distribution of ^{203}Hg labelled MeHg in mouse blood cerebral cortex and cerebellum at various times after intraperitoneal injection of 10 mg Hg/kg (each point represents the mean of 4 animals except for that at 168 hours which represents 2 animals)

levels of glycolytic intermediates which were found to increase with dose (see fig 1) exhibited a greater increase in the cortex than in the cerebellum, while the glycolytic intermediates and αGOP, which decreased with the dose, decreased more in the cerebellum The ratios of the changes in cortical levels to changes in cerebellar levels of adenine nucleotides and PC are all less than one, indicating greater changes in the cerebellum The only statistically significant regional differences were observed for αGOP, PYR and AMP ($P < 0\ 02$, 0 002 and 0 04, respectively)

Results of a study of the time dependent distribution of a 10 mg Hg/kg dose of ^{203}Hg labelled MeHg in the mouse brain and blood are shown in fig 3 It can be seen that the rate of decrease of ^{203}Hg in the blood is faster than the rate of uptake by the brain tissue The faster rate of accumulation by the cerebellum than the cortex at times up to 72 hours should be noted When the data for the first 4 time periods are analyzed by Student's t-test for paired data, the cerebellum exhibits a significantly greater uptake of ^{203}Hg than the cortex ($P < 0\ 0005$, average difference is 0 515 µg Hg/g tissue) Also the differential in the amount of ^{203}Hg found in the two brain regions decreases with time to 72 hours and is reversed by 168 hours When data for the differences in ^{203}Hg concentration between brain regions with time are analyzed by a one way analysis of variance, differences between the groups are highly significant ($P < 0\ 001$)

Behavioural Data for both dependent measures (rearings and ambulations) were summated across the ten minute observation period providing a total score for each animal These scores provided the basic data Data for each

measure were analyzed in a 2 factor, 3 × 4 factorial analysis of variance The factors were time (1, 3 and 72 hours) and dose (saline, 1, 5 and 10 mg Hg/kg)

The data for rearings are presented in fig 4A The analysis of variance yielded significant main effects both of time ($F = 24.88$, d f 2/108, $P < 0.001$) and dose ($F = 26.58$, d f 3/108, $P < 0.001$) as well as a significant time by dose interaction ($F = 5.89$, d f 6/108, $P < 0.001$) Individual comparisons between groups were made using the Duncan Multiple Range Test to determine the site of simple effects underlying the main effects The individual comparisons showed that the 1 mg Hg/kg dosage groups were not significantly different from the saline groups at any time period The 5 mg Hg/kg groups were significantly different from both the saline and 1 mg Hg/kg groups at both 1 and 3 hours after injection The 10 mg Hg/kg group was significantly different from all 3 other groups, both at 1 and 3 hours However, at 72 hours there was no difference among any of the groups Across time, the saline groups did not differ significantly from one

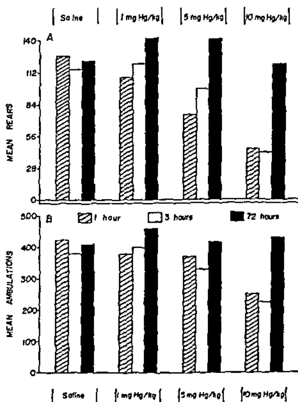


Fig 4 Mean number of rears and ambulations for each dose and time after each dose

another The 1 mg Hg/kg groups exhibited a tendency toward increased rearings across time while rearings in both the 5 and 10 mg Hg/kg groups increased dramatically (fig 4A) After 1 and 3 hours both the 5 and 10 mg Hg/kg groups were significantly lower than the saline groups, but, by 72 hours all mercury treated groups were at the saline level indicating complete recovery

The data for ambulations are presented in fig 4B The analysis of variance yielded significant main effects of both time ($F = 9\ 556$, d f 2/108, $P < 0\ 001$) and dose ($F = 8\ 108$, d f 3/108, $P < 0\ 001$) The interaction between time and dose was not significant although it approached significance ($F = 2\ 044$, d f 6/108, $P < 0\ 10$) Individual comparisons between groups showed that at 1 hour there were no differences between saline, 1 mg Hg/kg and 5 mg Hg/kg groups The 10 mg Hg/kg group differed slightly from all the other groups at 1 hour An identical pattern was found at 3 hours At 72 hours no significant differences among the groups could be detected indicating a complete recovery Across time, there were no differences in the saline, 1 or 5 mg Hg/kg groups The 10 mg Hg/kg group showed significantly lower numbers of rearings at 1 and 3 hours and a complete recovery at 72 hours

Discussion

Major effects of the experimental variables, dose and time on open field behaviour of the mouse have been observed These effects emerged most clearly from the data obtained on rearings The measure of ambulations was consistent with these findings, but equivalent levels of significance were not attained It should be noted that other studies on the effects of drugs on open field behaviour have indicated that rearings may be a more sensitive measure of behavioural alterations than total ambulations (ERIKSSON & WALLGREN 1967, MORRISON & LEE 1968) The dosage level had a major effect on behaviour The data clearly indicate significantly decreased rearings with increased doses of MeHg both at 1 and 3 hours after injection The ambulations data are consistent with these findings although only the 10 mg Hg/kg dose proved to be significantly different from the controls

Another key fact to emerge from the data is the change in the behaviour of all mercury treated groups across time The significant differences from the controls of the MeHg treated groups obtained at 1 and 3 hours after injection disappeared by 72 hours after injection across mercury dose By 72 hours, all treated groups had recovered completely These findings do not mean that the mercury concentrations used had only a marginal effect since 7 of 23 animals injected with 10 mg Hg/kg died within 4 days after injection

(note that 7 animals in the 1 hour groups were sacrificed immediately after running and thus were not included in the above statistics) None of the animals injected with the lower doses died At 72 hours all animals that had not died, behaved in a manner not significantly different from that of the controls *This finding is particularly interesting considering that the greatest* concentration of mercury is observed in the brain at approximately 3 days (fig 3)

Consideration of these time effects may elucidate the findings of POST *et al* (1972) and BROWN *et al* (1972) POST *et al* (1972), using rats, found no significant effects of mercury ingestion on 8 voluntary tasks in the open field, but their testing began 2 to 8 days after treatment ceased Similarly, BROWN *et al* (1972) found initial differences between post-weaning mercury treated mice and controls in a T-maze, but no differences in retest data conducted a week later It would seem that time after injection, for low doses of MeHg, is a critical factor to be considered in assesing behavioural effects

The results of the biochemical experiments show that MeHg exerts a dose related, time dependent, regional effect on levels of mouse brain PC, adenine nucleotides, αGOP and glycolytic intermediates

The increase in intermediates at the beginning of the glycolytic pathway (G6P, F6P, FDP) along with the decreased levels of PYR and αGOP indicate inhibition at some intermediate point in the pathway This apparent inhibition may be due to the interaction of MeHg with (1) glycolytic enzymes, (2) enzymes involved in adenine nucleotide metabolism resulting in an allosteric *inhibition of glycolysis* (LOWRY *et al* 1964, ROLLESTON & NEWSHOLME 1967, TAKAGAKI 1968) or (3) both of these possibilities Allosteric inhibition seems the more likely explanation in view of the fact that MeHg modifies brain adenine nucleotide levels (figs 1 and 2) and the Hg levels in brain tissue do not directly parallel the time dependent changes in glycolytic intermediates (compare figs 2 and 3)

Rat brain ATPase has been shown to be inhibited *in vitro* by low levels of MeHg (JACOBSON *et al* 1972) In this study, brain ATP levels were observed to increase in response to increased doses of MeHg suggesting that *ATPase is inhibited ATPase is a good candidate for MeHg inhibition* due to its location at the cell membrane, the site at which heavy metals would be likely to exert their toxic action (PASSOW *et al* 1961)

MeHg has also been shown to cause damage to the blood brain barrier *(CHANG & HARTMAN 1972b) which may effect brain metabolism profoundly*

It is also interesting to note that the changes observed in this study are qualitatively comparable to those observed in guinea pig cortex slices on addition of ATP to the incubation medium (TAKAGAKI 1968) PATTERSON & USHER (1971) have shown dose related alterations in rat brain levels of glycolytic intermediates and, less clearly adenine nucleotides in response to

MeHg Our results differ from theirs in several qualitative aspects This may be ascribable to species differences, different dose levels, observation times, brain regions or any combination of these factors The magnitude and time dependent course of MeHg induced changes in metabolite levels in mouse brain correlates with the observed behavioural alterations (figs 1, 2 and 4)

Thus, behaviour is effected more at 1 and 3 hours post MeHg administration than at 72 hours and the behavioural and metabolic alterations "wash out' with time more or less simultaneously However, mercury levels continue to increase in the brain up to 72 hours (fig 3) *This suggests that some* type of compensatory mechanism is set in operation to maintain brain metabolic and, therefore, behavioural homeostasis

The metabolic intermediates which exhibit statistically significant regional alterations induced by MeHg (αGOP, PYR and AMP) all exhibited greater changes in the cerebellum than in the cerebral cortex This is consistent with the behavioural data since the open field task is one measure of gross motor activity that is known to be largely controlled by the cerebellum This observation is also in good agreement with the differential uptake where the cerebellum exhibited a faster uptake of mercury from the blood than the cortex

It is to be noted that adenine nucleotides and PC are changed more in the cerebellum than in the cerebrum

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(note that 7 animals in the 1 hour groups were sacrificed immediately after running and thus were not included in the above statistics) None of the animals injected with the lower doses died At 72 hours, all animals that had not died, behaved *in a manner not significantly different from that of the* controls This finding is particularly interesting considering that the greatest concentration of mercury is observed in the brain at approximately 3 days (fig 3)

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Potassium-Efflux and the Response to Carbachol, Phenylephrine, Adrenaline, Noradrenaline, and Isoprenaline in Rabbit Antrum Muscle

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Abstract. Longitudinal strips from the antrum of rabbit stomachs were incubated for one hour in radioactive (^{42}K) Krebs solution and transferred to a constant flow apparatus where they were washed with non-radioactive solution. After an equilibration period of 28 min., the strips were exposed to 10^{-5} M carbachol or 10^{-4} M phenylephrine, noradrenaline, adrenaline or isoprenaline. Some carbachol treated strips were later also exposed to the adrenergic drugs. The mechanical responses and the effect of the drugs on ^{42}K efflux were studied. Carbachol which initiated rhythmic contractions or gave a "fused" (tetanic) contraction, produced a transient, statistically significant increase in K efflux as compared with untreated controls. Phenylephrine, noradrenaline, and adrenaline which had no effect or initiated rhythmic contractions in inactive strips, reduced the mechanical activity in strips previously exposed to carbachol. Isoprenaline had only inhibitory effects. All the four adrenergic drugs increased K-efflux significantly in inactive strips, probably due to α receptor stimulation, since the effect of isoprenaline could be blocked by phentolamine. Noradrenaline also increased K efflux significantly in strips exposed to carbachol. The increase in K efflux produced by phenylephrine in the antrum strips was not as great as that previously recorded in fundus strips. The K uptake was the same, but K efflux was greater than in the fundus. It is concluded that α receptor stimulation increases K efflux regardless of whether the mechanical response is excitatory, inhibitory, or nil.

Key words: K efflux – K uptake – adrenergic receptors – cholinergic receptors – *gastric smooth muscle – gastric motility.*

The mechanical activity of strips obtained from the antrum of rabbit stomachs is different from the mechanical activity of strips obtained from the fundus (HAFFNER 1971). In the antrum preparations rhythmic contractions dominate the motility-pattern, while the mechanical activity of the

fundus preparations consists mainly of slow, sustained alterations in tension Similar patterns of activity are obtained when the pressure-changes in the antrum and fundus of the rabbit, rat, and guinea-pig stomachs are studied (HAFFNER & STADAAS 1972, HAFFNER 1973b)

Stimulation of adrenergic α-receptors in circular rabbit *fundus* strips produces a slow sustained contraction, the magnitude of which depends both on the existing tone (HAFFNER 1972) and on the external K^+- and Ca^{++}-concentrations (HAFFNER 1973a) Associated with the contractile response a transient increase in K-efflux occurs (HAFFNER *et al* 1972), but this increase can be dissociated from the mechanical response by altering the external K^+- and Ca^{++}-concentrations (HAFFNER *et al* 1973)

Stimulation of the α-receptors in the *antrum* can give either an excitatory or an inhibitory mechanical effect, depending on the existing activity (HAFFNER 1971, HAFFNER & STADAAS 1972) Antrum strips therefore present a unique opportunity for studying in one preparation the alterations in K-efflux associated with both α-receptor mediated excitation, and α receptor mediated inhibition

In the present study we have investigated alterations in K-efflux associated with the mechanical responses to phenylephrine, adrenaline, noradrenaline, and isoprenaline in active and inactive antrum strips We have also compared the effect of phenylephrine (the most specific α-receptor stimulating agent used) on K efflux from the antrum, with that previously observed in fundus preparations In addition K-uptake has been determined, and compared with that in the fundus

Material and Methods

Fifteen male white rabbits weighing from two to four kg were killed by a blow on the head and bled by cutting the carotid arteries The stomach was removed and 10–14 longitudinal strips were prepared from the antrum The mucosa and most of the circular musculature were cut away in order to make the strips thin enough to fit easily into the constant flow apparatus This did not appear to affect the mechanical responses which were similar to those obtained previously in full thickness strips (HAFFNER 1971) All the strips were stored overnight in Krebs solution at 4° in order to make the pre investigation conditions as similar as possible On the next day the strips were incubated in aerated (95 % O_2 5 % CO_2) radioactive (^{42}K, 6 μci/ml initially) Krebs solution for one hour at 37° and mounted in a constant flow apparatus as described previously (HAFFNER *et al* 1972) As before they were washed with aerated Krebs solution at 37° which contained (mM) Na^+ 136 9 K^+ 5 9 Ca^{++} 2.5 Mg^{++} 1 2, HCO_3^- 15 5 $H_2PO_4^-$ 1 2, Cl^- 133.6 and glucose 11 5 (pH = 7 4) After passing through the constant flow apparatus the solution was collected in two minute samples The radioactivity of the samples and that remaining in the strips at the end of the experiments was determined in a Packard Auto-Gamma Spectrometer Model 2001

When the effects on inactive strips were studied the drugs were added 28 min after mounting and the strips were washed for a further 16 min period When the effects of phenylephrine, adrenaline noradrenaline and isoprenaline on preparations stimulated with carbachol were studied carbachol 10^{-5} M was added 28 min after mounting the adrenergic drugs were added 16 min later, and the strips were washed for another 16 min The effects of isoprenaline on preparations treated with phentolamine 5 3 × 10^{-5} M were tested on strips which had been exposed to phentolamine both in the incubation medium and from the start of the wash out

Drugs and concentrations used Carbacholine chloride (carbacholinum chloridum NFN) 10^{-6} – 10^{-5} M, phenylephrine HCl (metaoxedrinum NFN) 10^{-4} M, adrenaline bitartrate 10^{-4} M noradrenaline b tartrate 10^{-4} M, isoprenaline sulphate 10^{-4} M and phentolamine (regitin®, Ciba) 5 3 × 10^{-5} M The drugs were prepared each day from frozen stock solutions apart from phentolamine which was prepared from the commercially available 1 ml ampoules of regitin® Radioactive potassium (^{42}K) was supplied by Institutt for Atomenergi Kjeller, Norway

Calculations ^{42}K uptake was calculated by adding the radioactivity in all the samples of effluent to the radioactivity which remained in the preparation at the end of the experiment The fraction lost at any time during the experiment was determined by dividing the amount of tracer lost per min with the amount of tracer which the preparation contained at that time. The half time for K efflux for the slow part of the efflux curves was calculated from the difference in K efflux expressed as counts min^{-2} at 20 and 40 min The effects of the drugs were recorded as the maximal efflux 4–6 min after addition of the drugs expressed as per cent of the efflux value at the last interval before the drug was added (28 and 44 min after the start of the wash out) These values have been compared with the corresponding values for controls which were washed out without addition of drugs (inactive preparations) or which were washed out for 60 min., carbachol 10^{-5} M being added at 28 min (active preparations) Statistical significance was determined with Wilcoxon s two sample test (HODGES & LEHMANN 1964) α denotes level of significance

Results

Drug responses in inactive strips

Phenylephrine, adrenaline, noradrenaline, and isoprenaline all increased the K efflux from inactive antrum strips Table 1 shows the efflux values, number of preparations used, and statistical significance of difference from the controls The increase was greater after noradrenaline than after phenylephrine ($\alpha = 0\ 001$ for difference) and adrenaline ($\alpha = 0\ 06$) It should also be noted that the increase produced by isoprenaline 10^{-4} M could be blocked by phentolamine 5 3 × 10^{-5} M which indicates that the increase in ^{42}K-efflux produced by the adrenergic agents is due to stimulation of α receptors

Rhythmic activity was initiated in some strips by all the three α-receptor stimulating agents Adrenaline produced contractions in 3 out of 6 strips, noradrenaline in 2 out of 6 and phenylephrine in 2 out of 16 strips No contractions were seen after the addition of isoprenaline

Table 1

Altered ^{42}K efflux in inactive antrum strips

	Control	PE	A	NA	ISO	PA	PA+ISO
No tested	9	16	6	6	6	11	6
K efflux	93 4	96 7	100 7	112 05	97 2	97 9	92.0
Significance		0 03	0 006	0 0009	0 03	–*	–**

PE = phenylephrine 10^{-4} M A = adrenaline 10^{-4} M, NA = noradrenaline 10^{-4} M
ISO = isoprenaline 10^{-4} M, PA = phentolamine 5 3 × 10^{-5} M
PA + ISO = phentolamine 5 3 × 10^{-5} M + isoprenaline 10^{-4} M

* Not significantly different from Control

** Not significantly different from either Control or PA

No correlation was found between the mechanical effect and alteration in ^{42}K-efflux As shown in figs 1 and 2, the increase in ^{42}K-efflux in response to noradrenaline occurred both in the strips in which no mechanical effect was obtained, and in the strips in which noradrenaline initiated rhythmic contractions

Carbachol produced a statistically significant increase in ^{42}K-efflux, both in concentrations of 10^{-6} M (6 strips, median 109 %, $\alpha = 0\,02$), and 10^{-5} M (71 strips, median 126,8 %, $\alpha = 0\,0001$) Carbachol 10^{-5} M was used to

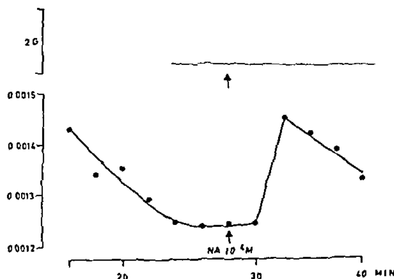


Fig 1 Addition of 10^{-4} M noradrenaline produces no mechanical response (top tracing) in an inactive antrum strip but increases ^{42}K-efflux markedly (bottom tracing = fraction lost curve)

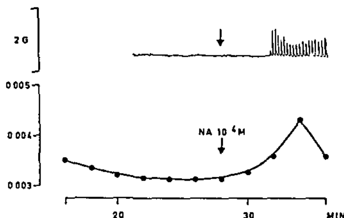


Fig 2 Addition of 10^{-4} M noradrenaline initiates rhythmic contractions (top tracing) in an inactive antrum strip, and also produces a transient increase in ^{42}K efflux (bottom tracing = fraction lost curve)

activate the strips in the present investigation as 10^{-6} M failed to initiate rhythmic contractions in one out of six strips in a preliminary series, while carbachol 10^{-5} M always gave contractile effects The mechanical response to carbachol usually consisted of an uninterrupted series of rhythmic contractions, but occasionally a brief period of rhythmic contractions was followed by a prolonged period of "fused' (tetanic) contraction (fig 3)

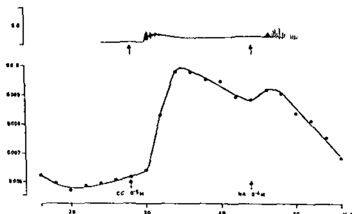


Fig 3 *Top tracing* Addition of 10^{-5} M carbachol to an inactive antrum strip initiates a burst of rhythmic contractions which progresses into a fused (tetanic) contraction (top tracing) Addition of 10^{-4} M noradrenaline reduces the fused contraction into a series of rhythmic contractions which gradually fade out *Bottom tracing* Both carbachol and noradrenaline increase ^{42}K-efflux (fraction lost curve)

Drug responses in active antrum strips

Noradrenaline was the only one of the adrenergic drugs which caused a statistically significant alteration in ^{42}K-efflux in the active strips The ^{42}K-efflux increased to a median of 110 7 % in 12 strips, as compared with 96 7 % in 8 controls ($\alpha = 0\ 01$ for the difference) No significant effects were obtained with adrenaline (13 strips), phenylephrine (19 strips) or isoprenaline (5 strips), although all the drugs produced inhibitory mechanical effects In the preparations in which a "fused" contraction had been produced by carbachol, the inhibitory effect could well be mistaken for an excitatory one, the "fused" contraction was altered into a series of rhythmic contractions of higher amplitude (fig 3), but it must be realized that both this effect and a reduction or total inhibition of rhythmic contractions (fig 4) represent inhibitory responses

Comparison of ^{42}K efflux after phenylephrine in antrum and fundus strips

The median increase in ^{42}K-efflux produced by phenylephrine 10^{-4} M in 16 inactive antrum strips was 3 3 % (median value of the increase in each strip after phenylephrine, less median value of controls) The increase in 12 inactive fundus strips tested in standard solution after one night in cold storage (HAFFNER *et al* 1973) was 11 5 % This difference is statistically significant ($\alpha = 0\ 022$) α-receptor stimulation therefore produces a greater increase in K-efflux in fundus than in antrum strips

No comparison was possible for active strips, since no studies have been

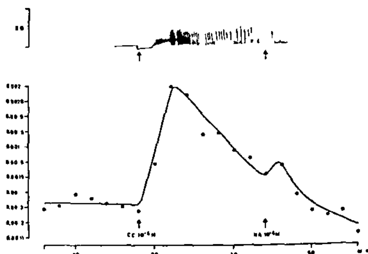


Fig 4 *Top tracing* Addition of 10^{-5} M carbachol to an inactive antrum strip initiates a series of rhythmic contractions which are inhibited by 10^{-4} M noradrenaline *Bottom tracing* Both carbachol and noradrenaline increase ^{42}K-efflux (fraction lost curve)

carried out on the alteration in K-efflux produced by phenylephrine in active fundus strips

Comparison of ^{42}K uptake and efflux in antrum and fundus strips

The median uptake of potassium in 8 control antrum strips was 144 1 $\times$ 10^{-6} mM/kg dry weight This is almost identical with the uptake previously found in fundus strips (HAFFNER *et al* 1973), i e 153 7 $\times$ 10^{-6} mM/kg dry weight

The median half time for the efflux in the antrum strips, calculated from the difference in efflux at 20 and 40 min of wash out, was 34 5 min, which is significantly lower ($\alpha = 0$ 031) than that in fundus strips under similar conditions, i e 43 1 min.

The median fraction lost at 32 min of wash out was 0 00902 in the antrum strips as compared with 0 00375 in the fundus ($\alpha = 0$ 08) The fraction lost values varied greatly, both in the fundus and antrum strips, possibly because the size and shape of the individual strips varied a little, giving different ratios of surface area/volume (See discussion for possible importance of difference in size of antrum and fundus strips)

These results show that the uptake of ^{42}K is the same in antrum and fundus strips, but that the antrum strips loose ^{42}K more rapidly than the fundus strips

Discussion

It has previously been reported that both excitatory (HAFFNER *et al* 1972 & 1973) and inhibitory (see DANIEL *et al* 1970 for ref) mechanical responses to α receptor stimulation are associated with a transient increase in K-efflux These studies were carried out on different preparations however, which either gave only excitatory or only inhibitory mechanical responses to α-receptor stimulation The differences in mechanical responses might therefore be due to differences in cellular organization or biochemical com position in the different preparations

In the present investigation the alteration in K efflux produced by adrenergic agents has been studied in a preparation which can give either an excitatory or an inhibitory mechanical response to α-receptor stimulation depending on the degree of existing activity when the receptors are stimulated The results show that α receptor stimulation increases K-efflux irrespective of whether the mechanical response is excitatory, inhibitory, or nil and irrespective of whether the preparation is active or inactive From these experiments and those previously published by us and other investigators, it may safely be concluded that the increase in K-efflux has no causal

relationship to the quality of the mechanical response The importance of the alteration in K-efflux is uncertain, both we (HAFFNER et al 1973) and others (BASS et al 1964) have been able to obtain contractile responses in smooth muscle preparations under conditions in which no increase occurs in K-efflux Furthermore, as K-efflux is increased irrespective of the quality of the mechanical response, it seems unlikely that increased permeability to K^+ is a necessary step in the mechanical α-receptor response Nor is it likely that the alteration in efflux is secondary to the mechanical response, if so, no alteration would have occured in the preparations which failed to give a mechanical response The increase in K-efflux therefore seems to be a concomitant result of α-receptor stimulation, but one which is not necessarily a link in the chain of events which produces the mechanical response

One possible explanation for the increase in K-efflux is that it is secondary to the alterations in membrane potential associated with mechanical responses to α-receptor stimulation (see KURIYAMA 1970) This seems quite likely since no increase in K-efflux was obtained in fundus strips submerged in high K^+-Krebs solution (HAFFNER et al 1973) which is known to depolarize the cell membrane almost completely The increase in K-efflux associated with excitation (membrane depolarization) may be part of a general increase in membrane permeability to several ions, while the increase in K-efflux associated with inhibition (membrane hyperpolarization) may be due to a selective increase in the permeability for potassium

In the present investigation it was found that the increase in K-efflux produced by α receptor stimulation is less marked in antrum strips than in fundus strips The difference could be due to differences in thickness, as the antrum preparations generally were thicker than the fundus preparations and therefore had a smaller surface area per unit volume It seems unlikely that this should be the explanation however, since the K-efflux from the antrum preparations was greater than from the fundus A more likely explanation is that the membrane properties of the smooth muscle in the antrum differ from those of the smooth muscle in the fundus The differences in type of mechanical activity in the two parts would seem to indicate this It is very unlikely that the difference should be due to differences in content of K^+ in the two parts of the stomach, since the K uptake was almost identical in preparations from the fundus and antrum

The K uptakes determined in the present and the previous (HAFFNER et al 1973) investigations are considerably lower than the K-uptakes found in other smooth muscle preparations, but comparison is difficult since we have determined the dry weight, while most other investigators have used wet weight CASTEELS (1970) reports that the wet weight/dry weight ratio is 18 for guinea-pig taenia coli, and 17 8 for the vas deferens If the same ratio i e 18 is correct for rabbit fundus and antrum preparations, the uptakes

would be approximately 8 mM/kg wet weight in one hour This is considerably lower than the uptakes in guinea pig taenia coli (= 72 mM, GOODFORD 1966, CASTEELS 1970), pregnant rat myometrium (= 42 mM, DANIEL *et al* 1970) and rabbit mesenteric vein (= 29 6 mM, MATTEWS & SUTTER 1967) The cause of this difference is unknown It may partly be explained by the fact that our preparations were not stretched while they were loaded with ^{42}K, contrary to the other investigators who studied uptake under isotonic conditions by applying a constant load to the preparations Another factor which may have influenced the K uptake is cold storage, our preparations were studied after cold storage over night, whereas other investigators have studied fresh preparations, and although BAUER et al (1963) failed to find any difference in K-uptake in fresh and 24 hour cold storage preparations of guinea pig taenia coli, effects of cold storage on stomach preparations cannot be excluded, especially since our previous investigation (HAFFNER *et al* 1973) showed that K-uptake after two nights cold storage was significantly different from the uptake after one night Some difference must also be expected due to different species and organs studied

In conclusion it may be said that the present investigation has shown that even in the same preparation, α receptor stimulation produces an increase in K efflux regardless of whether the mechanical response is excitatory, inhibitory, or nil The present investigation has also shown that α-receptor stimulation produces a significantly greater increase in K-efflux in fundus than in antrum muscle, and that the uptake is roughly the same in these two preparations, whereas K efflux differs, K^+ being lost more rapidly from the antrum muscle strip

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Study on the Hyaluronic Acid-Protein Complex, the Molecular Size of Hyaluronic Acid and the Exchangeability of Chloride in Skin of Mice before and after Oestrogen Treatment

By

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(Received September 7, 1972, Accepted March 16, 1973)

Abstract Hyaluronic acid (HA)-protein complex was extracted with 0.2 M-NaCl from homogenized skin of the back of mice. The complex was separated from unrelated proteins by ethanol precipitation followed by three times chromatography on CM Sephadex columns The composition of the complex isolated from mice treated with oestradiol differed significantly from the complex of the control mice, the ratio HA/protein was 3.5 times higher in the former than in the latter The molecular sizes of the HA from the two samples were compared by means of gel filtration on Sepharose 2B column Apart from a high molecular fraction containing 4 % of the HA from mice treated with oestradiol no difference was found between the elution patterns The exchangeability of the chloride in the skin of control mice and mice treated with oestradiol was studied by means of the isotope dilution method No significant difference was found between the two groups of mice

Key words Oestrogenic hormones – hyaluronic acid – protein complex – chloride – skin mice

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Subcutaneous connective tissue ground substance has, for several years, been assumed to function as a depot for water and electrolytes (Buddecke 1960, Hvidberg 1962, Langgård 1965a) The ground substance binds water and electrolytes in a three dimensional network made up of hyaluronic acid (HA) and proteins The system is highly organised and, most likely, the proteins are specific proteins which, in connection with HA, constitute well-defined HA-protein complexes forming the gel (Velican & Velican 1967)

Generally, HA and all the sulphated connective tissue polysaccharides are considered to be bound to protein in their native state in tissues (Schubert 1964, Schiller 1966) It seems well established that the chondroitin sulphates are covalently bound to protein(s) (Anderson *et al* 1965, Stern *et al*

1969, TSIGANOS & MUIR 1969, DEAN & MUIR 1970), whereas only a few studies have shown evidence of the existence of covalent bonds between HA and protein (HAMERMAN *et al* 1966, PRESTON *et al* 1965, WARDI *et al* 1969) The structure of the HA-protein complex has been the subject of only relatively few investigations and thus little information is available concerning this compound

The hypothesis that the ground substance exerts a depot function for water by means of HA is supported by the results of GROSMAN *et al* (1971), who found a linear correlation between the increase in water and HA content in the skin of mice treated with oestradiol (ovex mice)

The results of the chloride exchange experiments of LANGGÅRD (1965b) indicate that HA might also be involved in a chloride binding mechanism in the tissue He found that a part of the chloride exchanged at a low rate and the amount of slowly exchanging chloride were correlated to the tissue content of HA This finding could reflect a specific property of the tissue If this is the case, it seems likely that the binding of chloride is caused by HA-protein complexes

The purpose of the present study was, in continuation of previous works (GROSMAN *et al* 1971), to elucidate the changes induced by oestrogen treatment in the ground substance in the skin of mice The HA-protein complex from the skin of control and ovex mice was isolated and the possibility of a relationship between the content and composition of this compound and the exchangeability of the chloride was examined Further the degree of polymerization of the isolated HA was investigated by means of gel filtration on Sepharose® 2B column

Materials and methods

White male mice (NMRI/BOM strain) were treated with oestradiol monobenzoate and the skin was dried defatted and homogenized as previously described (GROSMAN *et al* 1971)

Extraction of HA from skin homogenate HA was extracted almost quantitatively with 0 2 M–NaCl 3 times 4 ml/100 mg dry fat free homogenate The extraction was performed at room temperature in plastic stoppered centrifuge tubes which were gently rotated for 1 hr/extraction Fractionation of the extract on cellulose columns according to the method by GROSMAN *et al* (1971) showed that HA was the only acid mucopolysaccharide isolated by this method The three extracts were pooled and HA precipitated by addition of 3 volumes of 96 % ethanol (SWANN 1968a) and left at room temperature over night

Deproteinization of HA The ethanol precipitation caused an irreversible denaturation of most of the extracted tissue protein The HA was dissolved in 0 02 M citrate buffer pH = 5 2 and further deproteinized by applying the supernatant to a CM Sephadex column 1 × 30 cm equilibrated with 0 02 M citrate buffer pH = 5 2 and eluted with the same buffer (BERMAN 1966) The eluate was collected in 5 ml fractions which

were analysed for uronic acid and protein The HA was mainly found in the third fraction but usually the second and fourth fraction were also found to contain some HA The HA containing fractions were rechromatographed twice on CM Sephadex columns A fourth passage through CM Sephadex showed that the ratio of uronic acid/protein was constant after the third passage indicating that only protein firmly "bound to HA was eluted

The recovery of a single run on CM Sephadex columns was close to 100 % The determination of the ratio HA/protein was performed on single ovex mice whereas extracts from four control mice were pooled before deproteinization on CM Sephadex columns

Gel filtration on Sepharose 2B HA was chromatographed on a Sepharose 2B column 2 5 × 40 cm equilibrated with 0 002 M phosphate buffer containing 0 1 M–NaCl pH = 7 0 the same buffer was used as eluant The column was run with an upward flow maintained at 9 ml/hr by a peristaltic pump and 5 fractions were collected per hr By means of Blue Dextran the void volume was determined to 74 ml and the total volume to 220 ml The column was not calibrated for molecular weight determination as the elution diagrams were only used for comparing the degree of polymerization of HA from control and ovex mice The ethanol precipitated extracts as well as the deproteinized material from CM Sephadex columns were used for the filtration experiment and identical elution diagrams were obtained

Hexuronic acid was determined by BITTER & MUIR s (1962) modification of the carbazole analysis with glucuronic acid as standard

Proteins were determined by MILLER s (1959) modification of the method of LOWRY *et al* (1951) with BSA as standard

Exchange of chloride The chloride exchange experiments were performed essentially by the procedure of LANGGÅRD (1965b) with minor modifications

20 µl of a 0 9 % NaCl solution containing 0 08 µci of ^{36}Cl as NaCl was injected into a tail vein and groups of animals were examined 10 min 1 hr or 23 hrs later One half of the skin was freeze-dried and defatted to determine the water content and the dry fat free weight of the tissue The other half was hydrolysed in 1 ml of 0 5 N–NaOH at 70° The proteins were precipitated by adding 1 ml 10 % $ZnSO_4$ Plasma samples were treated in an identical way i e to 100 µl plasma was added 300 µl 0 5 N–NaOH followed by 300 µl 10 % $ZnSO_4$ After centrifugation 100 µl of the clear supernatants was added to a scintillation medium described by BRAY (1960) and the radioactivity measured in a Packard Tri Carb liquid scintillation spectrometer

Another aliquot of the supernatant was used for chemical determination of chloride by automatic potentiometric titration with 0 02 M $AgNO_3$ (Radiometer's® titrator) 1 ml of tissue samples and 300 µl of plasma samples were used

Results

The composition of the HA-protein complex is shown in table 1 The ratio HA/protein is 3 5 fold higher in the complex isolated from ovex mice as compared with the complex from control mice When calculating the composition as the percentage of protein in the complex this value in ovex mice is found to be half of that of the control mice

Fig 1 shows two typical elution diagrams from Sepharose 2B column of the isolated HA from the skin of control mice (fig 1a) and ovex mice

Table 1

The composition of HA protein complex deproteinized on CM Sephadex columns

	g HA/g protein	% protein in the complex
Control mice (n = 7)	1 54 ± 0 04	39 5 ± 0 7
Ovex mice (n = 13)	5 32 ± 0 41	16 6 ± 1 1
	P < 0 001	P < 0 001

The values are given as the mean ± S E M The difference between control and ovex mice was tested by means of Student s t test

(fig 1b) The diagrams are divided into three parts, as indicated on fig 1 Material, which is excluded of the gel, is eluted with the first 93 ml of eluate with a peak at 74 ml of eluate (part I) Between 93 ml and 176 ml of eluate

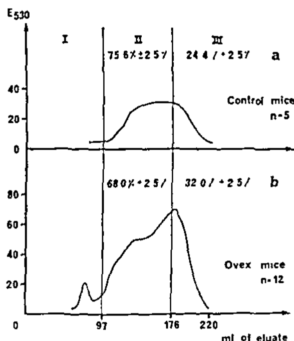


Fig 3 Elution diagram of HA from control mice (a) and ovex mice (b) on a Sepharose 2B column 2 5 × 40 cm The column was eluted with 0 002 M phosphate buffer containing 0 1 M NaCl pH = 7 0 flow rate 9 ml/hr, 5 fractions were collected per hr The uronic acid content of the fractions was assayed by the method of BITTER & MUIR (1962) The mean relative distributions of HA in part II and part III are given in the diagrams

Table 2

The percentage of chloride in the skin which exchanged with plasma chloride

	10 min	1 hr	23 hrs
Control mice	89 6 ± 1 3 (n = 31)	95 5 ± 2 5 (n = 15)	94 3 ± 2 2 (n = 4)
Ovex mice	88 2 ± 0 8 (n = 37)	99 0 ± 1 9 (n = 12)	
	P > 0 1	P > 0 1	

The values are given as the mean ± S E M The difference between control and ovex mice was tested by means of Student s t test

(part II) the material is eluted according to molecular size Material, the molecular dimensions of which allow it to disperse in the total volume of the column, is eluted between 176 ml and 220 ml of eluate with a peak at 187 ml (part III)

The elution diagrams of HA from ovex mice showed a peak in the void volume corresponding to 4 % of the eluted HA This fraction was not found in the control material Apart from that, no significant difference was found between the two types of elution patterns The mean relative distributions of HA were compared by means of Student's t test Both the difference of the distribution in part II and between part II and part III were tested resulting in P-values corresponding to 0 1

The results of the chloride exchange experiments are seen in table 2 The 10 min experiment is composed of three experiments performed on three independent groups of mice All results gave P > 0 1 when testing the difference between the control and ovex mice by means of Student s t-test Thus, it was not possible to reproduce the results of LANGGÅRD (1965b)

Discussion

The composition of the HA-protein complex from ovex mice differs significantly from the complex isolated from control mice (table 1) Following oestrogen treatment the tissue content of HA increases 5 4 times (GROSMAN *et al* 1971) and the ratio HA/protein 3 5 times Based on these two values the increase in the amount of protein complex-bound to HA is calculated to 1 5 fold

It cannot be completely excluded that the isolated HA-protein complexes are formed during the isolation procedures In a preliminary investigation

(GROSMAN, unpublished results) the HA protein complex was isolated by means of a different procedure (BERMAN 1962) The complexes isolated by the two procedures had identical ratio HA/protein which strongly indicates that they are of native origin This assumption is further supported by the different composition of the complex isolated from the skin of ovex mice as compared with the controls

As mentioned in the introduction only little is known about HA protein complexes HA and proteins might be synthesized separately and become at tached to each other later on, but they may also be metabolized as a unit It is not known whether a constant number of protein molecules are bound to each HA molecule or whether the amount of protein bound to HA depends on the molecular size of the HA molecule (HA is known to be polydisperse (LAURENT 1966))

The degree of polymerization of HA in the two tissues was compared by means of gel filtration on Sepharose 2B Only 4 % of the HA from ovex mice had a higher molecular weight whereas the remainder showed the same elution pattern as the HA from control mice Thus, differences in molecular size could not account for the different composition of the HA protein complexes

The different composition of the HA protein complex from the skin of control and ovex mice is more likely a consequence of the mechanism by which this compound is synthesized The experiment of DAVIDSON & SMALL (1963) suggested that there was more than one metabolic pool for HA in the skin This assumption was supported by the finding in the skin of mice (GROSMAN *et al* 1971) of a low molecular HA fraction, which increased 8 fold after oestrogen treatment, and by the isolation of four different fractions of HA from rooster combs (SWANN 1968a) SWANN (1968b) determined the protein content of the HA fractions to 0 35 % and despite large variations in molecular sizes the amino content per mol of uronic acid was very similar in the different fractions This investigation led to the hypothesis that HA contains at least one, but probably more, chemically bound protein or peptide component and that this molecule occurs in the connective tissues *as a complex with tissue proteins*

HA has a high metabolic activity, which is intensified by treatment with oestradiol It is a reasonable assumption that only some of the proteins in the complex – perhaps only the covalently bound proteins – are metabolized at the same rate as HA The metabolism of these proteins and HA is regulated by the same mechanism whereas the metabolism of the remainder of the proteins in the complex is controlled more or less independently This could be the reason for the small increase in the amount of complex bound protein as compared with the drastic increase in HA after oestrogen treatment Fur ther studies are needed to show the reliability of this assumption – involving

fractionation, characterization and quantitation of the proteins in the HA protein complex

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Effect of Guanethidine on the Ultrastructure of the Small, Granule-containing Cells in Cultures of Rat Sympathetic Ganglia[1]

By

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Abstract Sympathetic chain ganglia of newborn rats were cultured in Rose chambers with and without guanethidine After one week, the cultures were examined by light microscopy for formaldehyde induced catecholamine fluorescence and by electron microscopy after fixation in 5 % glutaraldehyde and 1 % osmium tetroxide Guanethidine sulphate (2 mg/l) caused an increase in the number of the small intensely fluorescent (SIF) cells in the ganglion explants Electron microscopic examination of guanethidine-containing cultures revealed an increased number of small granule-containing (SGC) cells, which corresponded in size and shape to the SIF cells Round vesicles (about 100 nm in diameter) and elongated vesicles (about 80 nm in cross section and about 200 nm in length) containing an electron dense core were observed in the cytoplasm of the SGC cells both in control and guanethidine containing cultures The granular vesicles were most frequent in the periphery of the cytoplasm In ganglia cultured with guanethidine, most SGC cells observed contained a greatly reduced number of granular vesicles as compared to SGC cells of the control cultures

Key words Guanethidine – small granule containing cells – cultures – ultrastructure

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Sympathetic ganglia are known to contain small, intensely fluorescent (SIF) cells amongst typical sympathetic nerve cells (ERÄNKÖ & HÄRKÖNEN 1963 & 1965, review by ERÄNKÖ & ERÄNKÖ 1971b) These cells correspond electronmicroscopically to small, granule containing (SGC) cells, which are characterised chiefly by the presence of cytoplasmic vesicles containing an electron dense core (MATTHEWS & RAISMAN 1969)

Prolonged administration of guanethidine has been shown to cause degenerative changes in neurons of the sympathetic ganglia of adult rats (JENSEN HOLM & JUUL 1970 & 1971, BURNSTOCK *et al* 1971, HEATH *et al* 1972, ERÄNKÖ & ERÄNKÖ 1971b) In newborn rats, guanethidine causes destruction of sympathetic nerve cells (ERÄNKÖ & ERÄNKÖ 1971a, ANGELETTI *et al* 1972) but a marked increase in the number of the SIF cells (ERÄNKÖ & ERÄNKÖ 1971a) A similar increase in the number of SIF cells has been observed in cultures of sympathetic ganglia containing a low or moderate concentration of guanethidine (ERÄNKÖ *et al* 1972a) The present study was undertaken to compare the ultrastructure of these cells in control cultures and in cultures containing guanethidine

Materials and methods

Newborn albino rats of the Sprague Dawley strain were killed by a blow on the head The thoracic and abdominal sympathetic chain was dissected with the aid of a binocular microscope under sterile cond tions carefully avoiding damage to the ganglia The chains were then treated with 0 125 % trypsin in a balanced salt solution and after cutting the connections the separated ganglia were transferred to collagen-coated coverslips

Details of the method of tissue culture have been described by CHAMLEY *et al* (1972) However it should be mentioned here that the cultures were carried out in modified ROSE (1954) chambers containing Medium 199 (SALK *et al* 1954) supplemented with 20 % (v/v) of foetal calf serum 0 05 units/ml of insulin 100 units/ml of penicillin G 5 mg/ml of glucose and 1 unit/ml of nerve growth factor (Burroughs and Wellcome England) Guanethidine sulphate (ismelin® Ciba Geigy Basle) was dissolved in the *balanced salt solution to make a stock solution containing 0 2 mg/ml* This was filtered through a millipore filter and added to the culture medium in a proportion of 1 99 thus obtaining a final concentration of 2 micrograms of guanethidine sulphate per ml culture medium or 2 mg/l The culture chambers were kept in an incubator at 37° supplied with a continuous flow of 5 % carbon dioxide in atmospheric air (Common wealth Industrial Gases Australia) bubbled through water Both guanethidine-containing and control culture media were changed daily

The material comprised 12 guanethidine containing chambers and 12 control cham bers each containing 4 explants The cultures were carried out for 7 days They were opened on the 8th day and 4 control and 4 guanethidine-containing chambers were prepared for fluorescence microscopy The remain ng 8 control and 8 guanethidine containing chambers were prepared for electron microscopy and one explant from each of these was selected for detailed analysis

For fluorescence microscopic demonstration of catecholamines the cultures were first dried on the coverslip overnight in a dessicator containing phosphorus pentoxide at room temperature They were then exposed to formaldehyde vapour from para formaldehyde powder, equilibrated with air of 60 % relative humidity in loosely closed jars at 50° for 30 min and subsequently at 80° for 1 hour They were then mounted in liquid paraffin and examined in a Leitz Ortholux fluorescence microscope with an HBO 200 mercury lamp and a filter combination of BG12 and K510 Further details on the histochemical method are given in previous papers (ERÄNKÖ 1967, ERÄNKÖ *et al* 1972b)

Material was prepared for electron microscopy by successive immersion in the following solutions at room temperature Cultures on the coverslips were fixed in 5 % glutaraldehyde in 0 1 M phosphate buffer (pH 7 4) for 30 min, washed in buffer for 10 min, post-fixed in 1 % buffered osmium tetroxide for 30 min, briefly rinsed in distilled water, stained in 2 % aqueous solution of uranyl acetate for 1 hour, dehydrated in a graded acetone series infiltrated in a mixture of equal parts of acetone and Araldite and finally embedded in Araldite Thin sections cut with a Huxley Cambridge ultramicrotome were double stained with a saturated aqueous solution of uranyl acetate followed by lead citrate (REYNOLDS 1963) and were subsequently examined with an Hitachi 11B electron microscope

Results

Catecholamine fluorescence

Control cultures SIF cells in the control cultures were usually found in small clusters, and exhibited a green fluorescence of low to moderate intensity Some single SIF cells were also seen Other areas of the ganglion explants exhibited only a weak diffuse fluorescence forming a background against which the SIF cell clusters stood out clearly

Guanethidine-containing cultures In guanethidine-containing cultures the clusters of SIF cells were larger and more numerous The colour of the fluorescence was green as in the control cultures, and the fluorescence intensity ranged from weak to moderate However, compared to control cultures, a greater number of weakly fluorescent SIF cells was observed

Ultrastructure

Control cultures The nuclei of SGC cells in control cultures showed dense chromatin aggregation, and the cytoplasm contained numerous mitochondria and abundant free ribosomes, usually in rosette formation Cisternae of rough endoplasmic reticulum and microtubules were present The granular vesicles, typically abundant in these cells, were mostly located in the periphery of the cytoplasm (fig 1) The granular vesicles were round (50–150 nm in diameter) or elongated (50–100 nm in width and 150–250 nm in length) in profile The central granule was usually of the same shape as the vesicle surrounding it, and an electron-lucent halo, usually 15–20 nm in width,

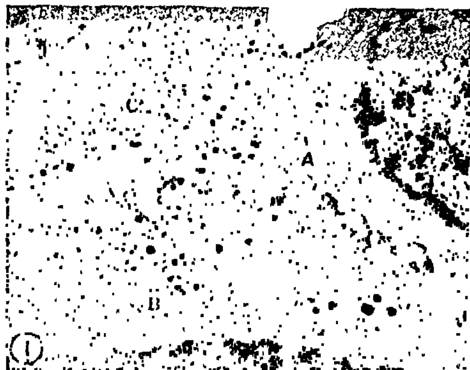


Fig 1 Typical appearance of SGC cells in control cultures The nuclei show dense chromatin aggregation, particularly in the periphery, and the cytoplasm contains an abundance of organelles The granular vesicles are located mainly in the periphery of the cytoplasm The cell membranes of cell A and cell B are in close contact while cell A and cell C are separated by a thin satellite cell process 7 day culture, Magnification × 22,000

was present between the granule and the vesicle membrane (fig 3) In some areas the outer cell membranes of the SGC cells were in close contact while in others a thin layer of satellite cell cytoplasm was observed between the SGC cells (fig 1)

Guanethidine-containing cultures The number of SGC cells observed was distinctly increased in all the guanethidine-containing cultures examined Although some variation existed between individual cultures, in approximately 75 % of observed SGC cells the population of granular vesicles was markedly reduced as compared to SGC cells of control cultures (fig 2) Many SGC cells in guanethidine-containing cultures contained only 10–20 granular vesicles per complete cell profile, compared to 100–250 granular vesicles per complete profile of a typical SGC cell of similar size in control cultures. The mitochondria in some SGC cells in guanethidine-con-

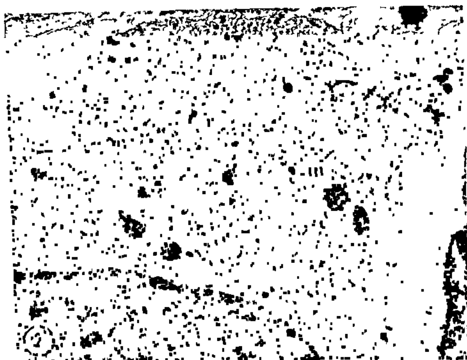


Fig 2 SGC cell in a guanethidine containing culture Only few granular vesicles are present in the cytoplasm, and the mitochondria (m) show damaged cristae and decreased density of the matrix. Otherwise the structure of the cell is similar to controls 7 day culture with 2 mg/l guanethidine sulphate included in the culture medium Magnification × 22,000

taining cultures (particularly those with greatest reduction of granular vesicles) showed structural damage to cristae, decreased density of the matrix and also swelling in some instances The ultrastructure of this group of SGC cells appeared to be otherwise normal. Some SGC cells (approximately 25 %) observed in guanethidine-containing cultures, however, appeared to be similar to typical SGC cells of control cultures in all ultrastructural aspects, including the population of granular vesicles (fig 4) The mitochondria of SGC cells in this minority group were similar in size to those in control SGC cells, and were always structurally intact It should be stated that a small number of observed SGC cells contained a number of granular vesicles intermediate between the two extremes described above, giving the impression that a continuum existed from the SGC cells with only few granules to those with numbers typical of SGC cells in control cultures

The dimensions and shapes of the granular vesicles in SGC cells of guan-



Fig 3 Control culture The granular vesicles of the SGC cell are round or elongated The granule takes the same shape as the bounding membrane An electron lucent halo is present between the membrane of the vesicle and its core 7 day culture Magnification × 35,000



Fig 4 Peripheral region of an SGC cell from a guanethidine containing culture The number of granular vesicles in this cell is comparable with that in SGC cells of control cultures The granular vesicles are of similar dimensions to those in control cultures and the cores of the vesicles are of similar electron-density to the controls (cf fig 3) Note *that the mitochondria are structurally intact. (The large size of some of* the mitochondria in this micrograph represents only an occasional finding in this specimen) 7 day culture with 2 mg/l guanethidine sulphate included in the culture medium Magnification × 35,000

ethidine-containing cultures were similar to those of control cultures, and there was no significant tendency towards either an increase or decrease in the size or electron density of the central core in SGC cells of the guanethidine treated cultures (fig 3, cf 4) Depleted or "empty" vesicles were not observed in SGC cells of guanethidine-treated cultures

Nerve cells and satellite cells appeared normal both in the control and the guanethidine containing cultures

Discussion

The increase in the number of SIF cells observed in the present study after the addition of 2 mg/l of guanethidine sulphate to the culture medium is in agreement with the previous observations that 1 and 3 mg/l of the same drug causes an increase in the number of SIF cells in cultures (ERÄNKÖ *et al* 1972a) and that guanethidine injections given to newborn rats result in a hyperplasia of these cells (ERÄNKÖ & ERÄNKÖ 1971a) The increase in the number of the SGC cells observed by electron microscopy in the present study is consistent with this increased number in the SIF cell population, since the SIF cells are believed to correspond to the SGC cells not only in sympathetic ganglia of normal adult (MATTHEWS & RAISMAN 1969, VAN ORDEN *et al* 1970, ERÄNKÖ & ERÄNKÖ 1971b) and newborn (ERÄNKÖ 1972) rats but also in cultures of sympathetic ganglia (LEVER & PRESLEY 1971, ERÄNKÖ *et al* 1972c)

The observation that the number of granular vesicles in most SGC cells of the guanethidine-containing cultures was less than that in SGC cells of controls is interesting, especially in view of the previous observation that an increase in the number of SGC cells during hydrocortisone treatment was associated with an *increase* in the number of granular vesicles in those cells both *in vitro* (ERÄNKÖ *et al* 1972c) and *in vivo* (ERÄNKÖ *et al* 1973) This suggests that the mechanisms by which hydrocortisone and guanethidine cause an increase in the number of SGC cells may be at least partly different It is possible that guanethidine may stimulate mitotic division of the SGC cells, this mechanism has been previously proposed following the observation that in the sympathetic ganglia of newborn rats injected with guanethidine the number of SIF cells in each cluster was greatly increased while the number of clusters was essentially unaffected (ERÄNKÖ & ERÄNKÖ 1971a) Electron microscopic observation of mitotic division of SGC cells in newborn rats has been reported by MASCORRO & YATES (1970) Although mitoses were not observed in the present study, these events could have occurred prior to the time of examination (after culture for 7 days) Increased formation of cate cholamine containing granular vesicles in primitive cells, presumably pre-

cursors of the SIF or SGC cells, has been proposed as a possible additional explanation for the apperance of numerous new SIF or SGC cells in newborn rats (ERANKO & ERANKÖ 1972, ERÄNKO *et al* 1973) and in cultures of sympathetic ganglia (ERANKO *et al* 1972b & 1972c) treated with hydrocortisone However, in the present study, this explanation would seem less likely since the fluorescence intensity and number of granular vesicles were decreased in many SIF or SGC cells That guanethidine causes at the same time an increase in the number of SGC cells and a loss in the average number of granular vesicles in them may be due to two separate actions of this drug, i e, stimulation of mitotic division of these cells and depletion of catecholamines from their storage granules Such mechanisms may explain the very variable reactions of the SIF cells observed in cultures containing high concentrations of guanethidine (ERANKÖ *et al* 1972a)

Studies of chronic high dose guanethidine treatment *in vivo* have shown that the mitochondria of adrenergic neurons, which selectively take up guanethidine, represent a primary site of action of this drug (JENSEN HOLM & JUUL 1971, HEATH *et al* 1972) Thus the mitochondrial damage observed in some SGC cells of guanethidine-containing cultures may represent a direct effect of this drug In this regard it is interesting that the SGC cells showing mitochondrial damage were those with the greatest reduction in the number of granular vesicles Since the concentration of guanethidine surrounding the ganglia in the present *in vitro* study is likely to be low compared to those which elicited damage in the *in vivo* studies (where doses of 10–40 mg/kg (JENSEN HOLM & JUUL 1971) and 30–100 mg/kg (HEATH *et al* 1972) were employed), this may explain why no ultrastructural sign of toxic effect was observed in the sympathetic neurons

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Biliary Excretion of Ampicillin, Azidocillin and Benzylpenicillin in the Rat

By

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Abstract The biliary excretion of intravenously administered ^{35}S-ampicillin ^{35}S azidocillin and ^{35}S-benzylpenicillin was studied in rats with ligated renal pedicles The total excretion of radioactivity was of the same magnitude for the three penicillins (65–72 % of the given doses) but the amounts of biologically active penicillin varied Of the injected dose 44 % of biologically active ampicillin was excreted into the bile while the corresponding values for benzyl penicillin and azidocillin were 19 % and 13 % respectively Chromatographic studies indicated that as well as the unchanged drug penicilloic acids of the corresponding penicillins were present in the bile Incubation studies with rat liver slices showed that ampicillin was more stable to degradation than benzyl penicillin and azidocillin It is suggested that the high hydrophobic character of the side chain of penicillins (high protein binding) may predispose to extensive biotransformation and lower amounts of unchanged penicillin in the bile

Key words Penicillins – biliary excretion

In most studies on biliary excretion of penicillins e g benzylpenicillin (ANDERSEN & BRODERSEN 1949) and ampicillin (STEWART & HARRISON 1961) only the biological activity has been assayed Therefore little information is available on the total excretion of penicillins (active and inactive penicillin) into the bile

Some new data indicate differences in bile concentration of biologically active penicillin after injection of various penicillins into rats (RYRFELDT 1971) These differences may be an expression of the differences in inactivation through biotransformation of various penicillins However, such factors as e g differences in affinity for transport system(s) and plasma protein binding may also be of importance (BANG & GEORG 1948)

The present investigation was undertaken in an attempt to obtain further information on these aspects by injecting ^{35}S labelled penicillins into rats

with bile fistulas and with ligated renal pedicles Uptake studies with rat liver slices were also performed The compounds used were two well known drugs, namely benzylpenicillin and ampicillin, and a new semisynthetic penicillin, azidocillin (SJOBERG *et al* 1967, HANSSON *et al* 1967)

Materials and methods

Chemicals

D-^{35}S ampicillin (spec act 0 6 and 40 μci/mg) ^{35}S benzylpenicillin (spec act 6 2 and 28 μci/mg) and D-^{35}S azidocillin (spec act 1 4 and 41 μci/mg) were obtained from the Research Laboratories Astra Lakemedel AB Sodertalje Sweden Benzylpenicillin and azidocillin were obtained as sodium salts and ampicillin was obtained in the amphoteric form The radiochemical purity of the compounds was checked by means of thin layer chromatography as described below The radiochemical purity of the compounds was about 95 %

Animal experiments

Male Sprague Dawley rats weighing 250–350 g were used The animals were anaesthetized with pentobarbital sodium (mebumalum NFN) (30 mg/kg intraperitoneally) and after laparatomy a polyethylene tube (PE 50) was inserted into the common bile duct The renal pedicles were ligated before the incision was closed Radioactive penicillins together with carriers were dissolved in physiological saline immediately before use and administered intravenously into the femoral vein The dose was 15 mg/kg calculated as the free acid of the compounds Before administration of the drug the bile flow was controlled and the flow was considered satisfactory if it exceeded 0 5 ml/hr During the experiment the animals were kept warm with a warming blanket The rectal temperature was recorded every quarter of an hour and maintained at 38 ± 1°

Bile was collected in preweighed sterile glass test tubes during two 30 minute periods During the sampling procedure the test tubes were chilled with crushed ice and during storage the samples were kept at −20°

Blood samples (about 0 5 ml) were taken from a polyethylene tubing inserted into the carotid artery at ½ hr and 1 hr after administration of the drugs The blood was collected in heparinized test tubes

At the end of the experiment the liver and kidneys were excised blotted free of blood and weighed These organs were homogenized in a Potter Elvehjem homogenizer w th an amount of water equal to twice the organ weight The homogenates were stored at −20° before analyses

Uptake studies

Male Sprague Dawley rats (175–200 g) were decapitated and the livers removed as quickly as possible They were placed in beakers containing ice cold saline Slices were cut out free hand (about 0 5 mm thick) placed in ice cold Tyrode s solution (pH 7 4 containing 1 g glucose/l) After this the slices were rinsed several times with ice cold Tyrode s solution and incubated for 10 minutes at 37° to get rid of weakly bound cell components

The uptake studies were performed in vials containing one slice (75–125 mg) and

1.00 ml Tyrode s solution and 1 00 ml serum or 1 00 ml Tyrode s buffer Pre incubation was performed for 10 minutes at 37° for temperature stabilization before 200 µl of saline, containing penicillin, was added The incubations were performed in an atmos phere carbogen (93 5 % O_2 and 6.5 % CO_2) and the vials were shaken in a metabolic shaker To stop the incubations the vials were taken from the incubator and placed in ice cold water The slices were removed, blotted on slightly moist filter paper and transferred to test tubes containing 1 00 ml ice cold water The slices were homogenized in a Potter Elvehjem homogenizer and the radioactivity of the homogenate as well as that of the medium was assayed

Results are expressed as slices to medium concentration ratios (S/M ratio) and are calculated as $\frac{\text{d p m /100 mg "slices"}}{\text{d p m /100 µl medium}}$.

Microb ological assay

Antibiotic concentrations in bile samples were determined by the cylinder plate biological assay method with Sarcina lutea ATCC 9341 as test organism (GROVE & RANDALL 1955) The dilutions for the standard curve were prepared in pooled rat bile and the bile samples were diluted in the same medium to give a concentration within the range of the standard curve

Blood samples were assayed by a micro method with filter paper discs (WALTER & MARGET 1958) Sarcina lutea ATCC 9341 was used as test organism The standard dilutions were prepared in rat whole blood Aliquots (10 µl) of these dilutions or samples were distributed to each of 6 discs (No 2668 Ø 6 mm, Schleicher & Schüll, Kassel Germany) To prevent coagulation a solution of Liquoid (Hoffman La Roche & Co AG) was used

Radioactive assay

Aliquots (100 µl) of water samples (bile and medium) were dissolved in 15 ml scintil lation solution (7 0 g Butyl PBD, 600 ml ethylene glycol monoethyl ether, 1000 ml toluene) The counting efficiency was estimated by the external standard channels ratio procedure and the counting performed so as to avoid an error larger than ± 3 % (95 % confidence limit) in the total count of each sample

The radioactivity of the tissue homogenates were obtained by dissolving 100 µl of a sample in 1 00 ml Soluene (Packard) and shaking the samples for 15 minutes After this 15 ml scintillation solution was added The counting was performed as described above

Chromatography

Pooled liver homogenates, representing the same incubation time, were centrifuged at 9000 r p m for 15 minutes at 0–4° and aliquots (1 00 ml) of the supernatants were freeze dried To the residue was added 0 20 ml of ice cold water and 20 µl samples were spotted on pre coated silicagel plates (E Merck AG) The plates were developed (10 cm) with n butanol acetic acid water (4 1 4, organic phase used) in sandwich chambers (Chemie Erzeugnisse und Absorptionstechnik AG, Switzerland) After devel oping and drying the plates, zones (one cm) were scraped off and the scrapings trans ferred to counting vials containing 0 05 M NaOH solution The vials were shaken for one hour, and after this scintillation solution was added and the samples assayed

Bile samples were also chromatographed as described above The radioactivity of these chromatograms was detected with a radio-chromatogram scanner (Packard Model

7200) and/or by pressing X ray film (Structurix D7, Gevaert) against the chromatograms for two three days The films were developed in Gevaert G 230 X ray developer for 5 minutes and fixed in Gevaert G 305 for 10 minutes

Enzymatic incubation

To 100 µl of bile samples (0–½ hr) 100 µl of a solution containing 1 mg penicillinase (neutrapen®, Riker) per 1 0 ml 1/15 M, sodium phosphate buffer pH 7 4 was added. *The incubations were performed at 37° for 1 hour*

Results

Excretion into the bile

The amounts of biologically active penicillin and radioactivity excreted into the bile are given in table 1 The excretion of radioactivity was of the same magnitude for the three penicillins during the 1-hour sampling period (65–72 %) However, the amounts of biologically active penicillins varied Ampicillin showed the highest excretion with 44 % of the given dose while benzylpenicillin and azidocillin were excreted by about 19 % and 13 %, respectively

Transfer of biologically active penicillin and radioactivity into the bile

In table 2 the concentration is given of biologically active penicillin in blood ½ and 1 hour after injection and in bile during the 0–½ and ½–1 hour intervals In this table the concentration of total radioactivity expressed as unchanged drug in the blood, bile and liver is also given

After injection of the penicillins a much higher concentration of biologically active penicillin was found in the bile samples than in the blood

Table 1

Cumulative excretion of biologically active penicillin and radioactivity in the bile after intravenous administration of ³⁵S-ampicillin ³⁵S-azidocillin and ³⁵S-benzylpenicillin (15 ml/kg body weight) into rats with bile fistulas and with ligated renal pedicles

Compound	Amount excreted (%)*			
	0–½ hr		0–1 hr	
	Biol act	Radiac	Biol act	Radiac
Ampicillin	30 3 ± 7 8	39 3 ± 6 3	43 6 ± 11 5	65.2 ± 7 0
Azidocillin	11 7 ± 2 8	44 9 ± 9 8	12 9 ± 2 3	64 6 ± 8 6
Benzylpenicillin	15 8 ± 0 8	49 3 ± 1 3	18 7 ± 1 5	71 6 ± 5 2

* Each value represents the mean ± S D for 3–4 experiments

Table 2

Concentrations of biologically active penicillin and total radioactivity expressed as unchanged drug in biological samples after intravenous administration of ³⁵S-ampicillin, ³⁵S azidocillin and ³⁵S benzylpenicillin (15 mg/kg body weight) into rats with bile fistulas and with ligated renal pedicles Bile was collected during two 30-minutes periods and blood was taken at the end of each bile collection period

Compound	Sample	Concentration (μg/g)* ½ hr		1 hr	
		Biol act	Total radiac	Biol act	Total radiac
Ampicillin	Bile	2093 ± 312	2329 ± 128	1172 ± 351	1933 ± 90
	Blood	2 8 ± 0 3	9 0 ± 1 8	2 3 ± 0 3	8 3 ± 1.2
	Liver	–	–	–	73 5 ± 11 6
Azidocillin	Bile	772 ± 64	2315 ± 206	112 ± 103	1257 ± 264
	Blood	2 3 ± 0 9	8 7 ± 0 4	1 1 ± 0 4	5 9 ± 0 2
	Liver	–	–	–	36 6 ± 12 8
Benzylpenicillin	Bile	939 ± 5	3127 ± 170	238 ± 51	1178 ± 26
	Blood	3 4 ± 0 9	11 0 ± 1 4	1 8 ± 0.2	8 0 ± 1.2
	Liver	–	–	–	33.2 ± 3 4

* Each value represents the mean ± S D for 3–4 experiments

samples In comparing the concentration found in the bile during the ½–1 hour interval with the mean concentration in blood obtained from the ½ and 1 hour samples, the concentration ratio was 451 for ampicillin and the corresponding values for benzylpenicillin and azidocillin were 92 and 56, respectively

In a similar comparison with total radioactivity in the bile and blood the values were 222 for ampicillin, 187 for benzylpenicillin, and 172 for azidocillin The liver concentrations of radioactivity were between the bile and blood concentrations Negligible amounts of radioactivity were found in the kidneys, indicating that the ligations had been adequate

Uptake of penicillins in liver slices

The results from the uptake studies are shown in fig 1 In the experiment without serum in the medium, the highest uptake of radioactivity was noted with azidocillin Benzylpenicillin showed a somewhat higher uptake than ampicillin After 60 minutes incubation the S/M-value for azidocillin was 3 84 ± 0 25 ($\bar{x}$ ± S D) while the corresponding values for benzylpenicillin and ampicillin were 1 96 ± 0 21 and 1 70 ± 0 32, respectively After 15 minutes incubation the S/M values of unchanged penicillin were about 0 7 for the three penicillins Sixty minutes incubation gave a S/M-value of about 1 0

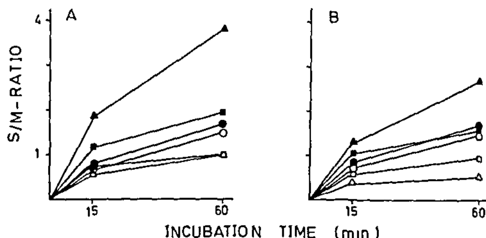


Fig 1 The uptake of penicillins, expressed as slices to medium concentration ratios (S/M ratio) by rat liver slices Incubations were performed without (A) and with serum in the medium (45 %) (B) Medium concentration of penicillin was 1 × 10^{-4} M Each point is the mean of 5 experiments Filled symbols represent total radioactivity and open symbols represent unchanged drug

△ azidocillin
□ benzylpenicillin
○ ampicillin

for benzylpenicillin and azidocillin but the corresponding value for ampicillin was 1 5

Serum in the incubation medium (45 %) could influence the uptake In the incubation studies with azidocillin the uptake of radioactivity was significantly decreased The 60 minutes value was 2 71 ± 0 29 compared with 3 84 without serum in the medium ($P < 0\ 001$) Benzylpenicillin showed a slight decrease (S/M 1 59 ± 0 33) compared with the control (S/M 1 96) but the ampicillin uptake (1 64 ± 0 21) was not influenced as compared with the control (S/M 1 70) The S/M-value of unchanged drug after 15 minutes incubation was about 0 8 for ampicillin and 0 5 for benzylpenicillin and azidocillin, respectively

The S/M-value of unchanged ampicillin after 60 minutes incubation was 1 4 while the corresponding values for benzylpenicillin and azidocillin were 0 9 and 0 5, respectively

Serum in the incubation medium did not influence the degradation of penicillin in the slices After 60 minutes incubation 88 % of the total radioactivity in the slices could be attributable to unchanged ampicillin when serum was present in the medium and with no serum in the medium, the value was 85 % The corresponding values for benzylpenicillin were 54 % and 56 % and for azidocillin 25 % and 20 %

Chromatographic studies of bile samples

After chromatography of bile obtained during the 0–½ hour interval after injection of azidocillin the activity was mainly localized to two spots with Rf values of 0 78 and 0 51 In the ½–1 hour bile sample nearly all of the activity was found in the spot with an Rf-value of 0 51 Authentic azidocillin had an Rf value of 0 78

After chromatography of bile samples obtained from benzylpenicillin treated rats, a distinct spot with the same Rf value as authentic benzylpenicillin (0 75) of the autoradiogram was found Other spots with Rf-values 0 45, 0 25, and 0 19 were also found The main spot of these was the one with an Rf-value of 0 45

The autoradiogram showed 3 radioactive spots with Rf-values of 0 45, 0 25, and 0 11 after chromatography of the bile samples obtained from rats treated with ampicillin The main spot had an Rf-value of 0 45 both in the 0–½ hour and ½–1 hour samples and this Rf value was the same as that of authentic ampicillin

After penicillinase treatment and chromatography of bile samples (0–½ hour) nearly all the activity could be found in one spot, for the respective penicillin, and this spot had the same Rf-value as the penicillinase treated solution administered For azidocillin the Rf-value was 0 51, for benzylpenicillin 0 45, and for ampicillin 0 25

Discussion

The present investigation shows that the excretion of biologically active penicillin varied markedly Of the injected dose, 44 % of active ampicillin was excreted into the bile, while the correseponding values for benzylpenicillin and azidocillin were 19 % and 13 %, respectively The fact that ampicillin showed a higher excretion into the bile than e g benzylpenicillin is in agreement with the results of HARRISON & STEWART (1961) and RYRFELDT (1971) The excretion of total radioactivity was about the same (65–72 %) for the three penicillins in our study

The reason for the differences in excretion of active penicillin into the bile may be due to inactivation through biotransformation, affinity for transport system(s) and/or plasma protein binding of the penicillins The high amounts of ampicillin excreted into the bile as compared with benzylpenicillin and even more so for azidocillin, may indicate, that ampicillin is more stable to inactivation (e g in liver) than the other two penicillins In support of this the results from the incubation studies with liver slices may be taken These experiments showed that ampicillin was more stable to degradation

than benzylpenicillin which was shown to be more stable than azidocillin KIND *et al* (1968) have also shown that ampicillin was more stable to degradation than benzylpenicillin, in rat liver perfusion studies It is noteworthy that azidocillin which shows the greatest degradation has the highest partition coefficient (log P) (RYRFELDT 1971) Such values can be used to describe the hydrophobic character of the side chain of the penicillins (BIRD & MARSHALL 1967) Since BIRD & MARSHALL have demonstrated a correlation between the hydrophobic character and protein binding (high log P-values – high protein binding), our results may suggest that azidocillin has a greater affinity for the enzyme(s) responsible for the degradation than benzylpenicillin and ampicillin

The concentration difference of biologically active penicillin between blood and bile which has been reported by investigators e g KIND *et al* (1968) and ANDERSON & BRODERSEN (1949), was also found in our study The bile to blood concentration ratio of biologically active penicillin as well as of total radioactivity was as much as 100 fold This indicates a concentrative transfer from blood to bile Our results also showed that the ratio of biologically active penicillin to total radioactivity was about the same for the three penicillins in the blood (0 31–0 32) but in the bile a higher ratio was found for ampicillin (0 90) than for azidocillin (0 33) and benzylpenicillin (0 30) This may be taken as an indication that ampicillin has a higher affinity for a transport system directed into the bile than the other two compounds The nature of such a transport has not been investigated here

The significance of plasma protein binding in the excretion of organic compounds has been a matter of debate BANG & GEORG (1948) suggested that compounds showing extensive binding to plasma proteins, are preferentially excreted in the bile while those showing low protein binding are mainly excreted in the urine That high plasma protein binding *per se* should further extensive biliary excretion does not seem to be very probable. However, a high affinity for proteins e g located intracellularly and in the membrane of the liver parenchyma may promote biliary excretion of organic compounds (CORNELIUS *et al* 1967, LEVI *et al* 1969) A more probable effect of plasma protein binding is a retarded excretion process The incubation studies with liver slices are indications of this The uptake rate of azidocillin was significantly reduced when serum was present in the incubation medium as compared with the control experiments The plasma binding of the penicillins used in this investigation were, ampicillin 18 %, benzylpenicillin 61 % (BIRD & MARSHALL 1967) and azidocillin 84 % (SJOBERG *et al* 1967) (all human data) BRAUER & PESSOTTI (1949) showed a similar effect on sulphobromophthalein excretion in liver perfusion experiments

In conclusion is suggested that a low hydrophobic character (low protein binding) predisposes to extensive biliary excretion of active penicillin A high hydrophobic character (high protein binding) promotes biotransformation and consequently small amounts of active penicillin are excreted in the bile

The chromatographic studies showed that metabolites were found in the biological samples, in addition to intact penicillin Incubation studies with penicillinase, followed by chromatography indicated that one of the metabolites, is probably the penicilloic acid of the corresponding penicillin However, this has to be confirmed with more specific methods than those used in this investigation The nature of the other metabolites found is not known but e g hydroxylation of benzene ring may occur (VANDERHAEGHE *et al* 1963)

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Components of the Kallikrein-Kinin System and the Spontaneous Cold Activation of Factor VII in Human Plasma

By

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Abstract Samples of plasma with the property of spontaneous activation of factor VII on incubation overnight at 0° were studied with regard to the concentration of prekallikrein kininogen fractions and the activity and concentration of proteinase inhibitors A significantly higher concentration of prekallikrein was observed in the cold activation positive plasmas from pregnant and control subjects The concentration of kininogen substrate 2, which was increased during pregnancy and treatment with oestrogenic oral contraceptives was not correlated with the spontaneous cold activation The rate of inhibition of kininogenase activity in normal plasma was very low at 0° as compared with that at 25°, and it is concluded that this may explain the odd temperature dependence of the spontaneous activation of factor VII

Key words Kallikrein – factor VII – cold – contraceptives, oral

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In 1967 Schrogie *et al* described spontaneous shortening of the thrombotest clotting time after storage overnight at 4–6° of plasma from women treated with oral contraceptives. Gjønnaess (1973) subsequently observed similar changes in plasmas from women in the last trimester of pregnancy and occasionally even in plasmas from untreated adults. He ascribed the cold promoted shortening of the thrombotest time to an activation of factor VII and also suggested that the cold activation might be caused by plasma kallikrein (Gjønnaess 1972b)

In the present work experiments were undertaken to determine whether the cold promoted activation of factor VII is related to changes in the kallikrein kinin system of plasma, and why the activation takes place at low temperature only

Materials and methods

All chemicals were of analytical grade Semicarbazide hydrochloride (Sigma Chemical Co, St Louis, Mo, U S A) was dissolved in twice distilled water and evaporated at 10 mm Hg at 60° to remove traces of ethanol Glassware and cannulas were siliconed (siliclad®, Clay Adams Parsippany, N J U S A) and plastic tubes were used to avoid contact activation of plasma

Blood was drawn from women in the third trimester of pregnancy from women [illegible]

1 5 mg (sekvens®, E Lilly Co, Ind U S A) and from untreated normals Plasma was obtained from blood collected into 1/10 volume of 0 1 M sodium citrate dihydrate by centrifugation at 3,100 × g for 30 minutes at 12° and stored at −20° Pooled plasma from three healthy men was stored in small aliquots at −20° and served as a reference plasma for the preparation of acetone activated plasma kallikrein and the 60° heated kininogen substrate

Preparations of kallikrein

A crude preparation of plasma kallikrein was prepared by activation of plasma with 16 7 % (v/v) acetone at room temperature for 17 hours and evaporation of the acetone A partially purified preparation of plasma prekallikrein was kindly supplied by A M Vennerød The purification of this preparation was 70 × and it yielded 1 8 BAEe esterase units per ml when activated with kaolin treated plasma (fig 3) On activation less than 5 % of the BAEe esterase and kininogenase activity in the incubate could be attributed to the kaolin activated plasma The specific activities of the preparations of kallikrein used were Crude plasma kallikrein 0 009 units/mg towards BAEe (1 mM) and 0 015 units/mg towards TAMe (10 mM) Activated prekallikrein 0 39 units/mg towards BAEe (1 mM) and 0 77 units/mg towards TAMe (10 mM) None of the preparations were active towards l lysine ethyl ester and N acetyl l tyrosine ethyl ester

Hog pancreas kallikrein for the determination of kininogen was obtained as depot padutin® R 100 (Farbenfabriken Bayer Leverkusen Germany) and dissolved to 40 biological units per ml in 0 15 M sodium chloride

Inhibition of plasma kininase

The plasma kininase was inhibited by incubation for 30 minutes at 37° with ethylene diamine tetraacetic acid disodium (EDTA 2Na) final concentration 4 mg/ml

Preparations of kininogen

A crude preparation of kininogen was prepared by heating of the reference plasma which had been treated with EDTA 2Na at 60° for 1 hour to deplete it of prekallikrein and factor XII

Determination of esterase activity

The rate of hydrolysis of α N benzoyl l arginine ethyl ester (BAEe Sigma) was estimated as described by TRAUTSCHOLD & WERLE (1961) with some modifications The reaction was run at 25° in cuvettes with 2 4 ml buffer at pH 8 8 (75 mM sodium pyrophosphate 75 mM semicarbazide hydrochloride and 50 mM glycine), 0 L ml 30 mM β nicotinamide adenine dinucleotide (Sigma) and 0 02 ml crystalline suspension of alcohol dehydrogenase from yeast (Boehringer Mannheim GmbH Germany) Finally 0 5 ml of a 6 mM solution of BAEe was added and the reaction was initiated with 0 03 ml of the sample After pre incubation for 5 minutes O D 340 was read

three to five times with a Hitachi 101 spectrophotometer combined with a W+W electronic 3012 recorder If the increase in optical density exceeded 0 03 O D per minute the sample volume was reduced Samples taken at intervals after kaolin activation of plasma showed constant esterase activity for more than 30 minutes indicating that no inhibition of the esterase activity took place in the cuvettes at this dilution of the contact activated plasma The results were corrected for spontaneous hydrolysis of BAEe, and the activity was transformed into enzyme units per ml One unit corresponded to μmole βAEe split per minute at 25°

The concentration of ethanol was determined by the same method, omitting BAEe and adding 0 5 ml of the sample instead O D 340 was read immediately and after in cubation for 30 minutes at 25°

Determination of kinin

The concentration of kinin was evaluated as bradykinin by bioassay on the isolated "estrus' rat uterus The samples were diluted 1 25 with 0 15 M sodium chloride at 100° and kept at this temperature for 5 minutes before the assay (BRISEID *et al* 1968)

Coagulation tests

The thrombotest (OWREN 1959) was performed in duplicate as recommended by the manufacturer (Nyegaard & Co, Oslo Norway) but with plasma instead of blood All the plasmas had initial clotting times between 35 and 45 seconds on thrombotest They were classified as being cold activation positive if the thrombotest clotting time after incubation at 0° for 20 hours was shorter than 20 seconds and as cold activation negative if the clotting time had not been shortened by more than 5 seconds on incubation in the cold

Immunological determination of proteinase inhibitors

The concentrations of C1 inactivator, α₂-macroglobulin, α₁ antitrypsin and anti thrombin 3 were determined by single radial immunodiffusion in 1 % (w/v) agarose in 0 07 M veronal buffer pH 8 6 (MANCINI *et al* 1964) The wells were circular, with a diameter of 2 1 mm and contained 0 01 ml samples The concentrations of antiserum in the agarose were as follows C̄1 inactivator 3 3 % (Behringwerke A G Marburg Lahn Germany) α₂-macroglobulin 1 3 % and α₁ antitrypsin (from Dr Fagerhol s laboratory) and antithrombin 33 3 % (Nyegaard & Co)

Statistics

The statistical significance of the data was tested by Student s one tailed t test

COMMENTS ON THE TECHNIQUE

Estimation of prekallikrein

COLMAN *et al* (1969a) have provided evidence that the arginine esterase activity after contact activation of plasma with kaolin is caused by plasma kallikrein The maximum esterase activity that is obtained with kaolin (final concentration 5 mg/ml) has been reported to reflect the concentration of prekallikrein (COLMAN *et al* 1969b) In our ex periments the esterase activity towards BAEe reached a maximum after incubation with kaolin for 30 seconds at 37° (fig 2), and this activity was taken to represent the concentration of prekallikrein in the plasmas after correction for the spontaneous esterolysis

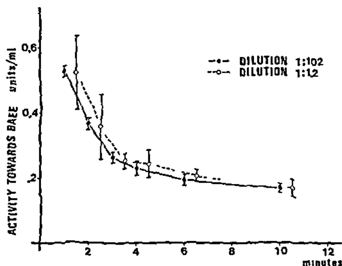


Fig 1 Inhibition of the kaolin activated esterase at 25° assayed at two different dilutions Plasma was activated by incubation with an equal volume of kaolin 10 mg/ml in 0 15 M sodium chloride At dilution 1 102 of the reaction mixture, 0 03 ml of the kaolin activated plasma was taken out at intervals and the esterase activity towards BAEe determined At dilution 1 1 2, 2 0 ml of the kaolin activated plasma was added to 0 4 ml 6 mM BAEe at intervals After 1 minute the esterolysis was stopped with 0 24 ml 100 % (w/v) trichloroacetic acid The mixture was then centrifuged and the ethanol was determined after retitration of the supernatant with 1 N sodium hydroxide to pH 8 0–9 0 Each point represents the mean of five observations with 95 % confidence interval

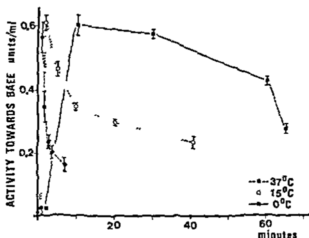


Fig 2 Contact activation and inhibition of plasma arginine esterase at different temperatures Plasma was activated by incubation with an equal volume of kaolin 10 mg/ml in 0 15 M sodium chloride, and the activity towards BAEe was recorded at intervals Each value represents the mean of 6 observations with 95 % confidence interval

Kinetic determination of proteinase inhibitors

After kaolin activation of plasma at 37° and 25° there was a rapid decrease in the activity towards BAEe to about 30 % of the peak activity (figs 1 and 2) C$\bar{1}$ inactivator inhibits plasma kallikrein (KAGEN 1964) and the initial reduction of the esterase activity after contact activation has been attributed to this inhibitor (COLMAN *et al* 1969b) According to HARPEL (1970), α_2-macroglobulin in plasma also reacts with plasma kallikrein and forms a complex with retention of about 50 % of the esterase activity Usually the assays for arginine esterase activity involves considerable dilution of the sample, and it might be that the residual activity observed after activation with kaolin merely represents enzyme activity which is desorbed from the kaolin, or dissociation of enzyme-inhibitor complexes at high K_I values of the inhibitors. If this were true, different kinetics for the inhibition of esterase activity after kaolin activation would be demonstrated in experiments with less dilution of the samples Fig 1 demonstrates that similar kinetics were observed when the samples were diluted 1 1 2 and 1 102 Thus the residual esterase activity on contact was probably caused neither by desorption from the kaolin nor by dissociation of enzyme inhibitor complexes on dilution

The initial loss of activity after kaolin activation was exponential towards an asymptote which represented the residual activity (fig 1) Plasma kallikrein activated by contact is inhibited by both C$\bar{1}$ inactivator and α_2-macroglobulin, the latter forming an esterase active complex with kallikrein The residual activity was calculated in per cent of the concentration of prekallikrein and used as an estimate for the concentration of C$\bar{1}$ inactivator relative to α_2 macroglobulin Pseudo first order reaction kinetics were applied on the exponential decay of the esterase activity at 37°, and the rate constants were taken to represent mainly the effect of C$\bar{1}$ inactivator on the contact activated esterase (COLMAN *et al* 1969b)

Results

1 Estimation of prekallikrein

Cold activation positive plasmas contained higher concentration of prekallikrein (P = 0 05) than the cold activation negative plasmas (table 1), but in the plasmas from women treated with oral contraceptives the difference was not statistically significant

2 Activity and concentration of plasma proteinase inhibitors

The kinetic data obtained after kaolin activation of cold activation positive and cold activation negative plasmas were completed with the immunological determinations of C$\bar{1}$-inactivator, α_2-macroglobulin, α_1-antitrypsin, and antithrombin 3 (table 2) The differences between the cold activation positive and negative plasmas within each group were not statistically significant when recorded either by the immunological or by the kinetic methods, even though the concentration of α_2 macroglobulin was somewhat lower in the cold activation positive plasmas

Table 1

Concentration of prekallikrein in plasma related to the spontaneous cold activation

Group	Pregnancy		Contraception		Untreated	
Cold activation	Positive	Negative	Positive	Negative	Positive	Negative
Concentration of prekallikrein U/ml	0 86 ± 0 10 (10)	0 69 ± 0 07 (5)	0 70 ± 0 09 (9)	0 55 ± 0 09 (5)	0 81 ± 0 05 (7)	0 60 ± 0 06 (5)

The results are given as the mean with 95 % confidence interval Number of experiments in brackets Significantly different results are underlined

Table 2

Kinetic and immunological estimation of the proteinase inhibitors in plasma related to the spontaneous cold activation

Group	Pregnancy		Contraception		Untreated	
Cold activation	Positive	Negative	Positive	Negative	Positive	Negative
Kinetic						
Rate min $^{-1}$*	−1.7 ± 0.5 (5)	1.7***	−1.7 ± 0.5 (5)	−1.7 ± 0.5 (5)	−1.1***	−1.6 ± 0.7 (5)
Residual activity %**	30 ± 7.4 (4)	32***	27 ± 10.6 (4)	21***	31***	23 ± 6.3 (5)
Immunological						
C1 inactivator %	75 ± 6.0 (21)	70 ± 5.9 (4)	88 ± 5.6 (32)	97 ± 10.3 (14)	102 ± 13.3 (8)	109 ± 4.5 (41)
α_2-macroglobulin %	114 ± 11.1 (24)	130 ± 35.3 (4)	103 ± 8.3 (32)	116 ± 21.2 (14)	88 ± 12.2 (8)	102 ± 5.4 (72)
α_1-antitrypsin %	155 ± 7.3 (24)	153 ± 15.0 (4)	124 ± 5.7 (32)	120 ± 9.6 (14)	83 ± 5.3 (8)	89 ± 3.7 (72)
Antithrombin 3 %	114 ± 5.8 (24)	101 ± 13.6 (4)	124 ± 5.3 (32)	115 ± 9.0 (14)	125 ± 8.7 (8)	130 ± 3.2 (72)

The results without asterisks represent the means with 95 % confidence interval

Number of experiments in brackets

* The rate of inhibition of the contact activated esterase activity is given as the first order rate constant of the decay of the esterase activity at 37°

** The residual activity is given in per cent of the prekallikrein concentration

*** The mean of two observations

3 Inhibition of plasma kallikrein in human plasma at low temperature

The spontaneous cold activation of factor VII takes place below 8° only (GJONNAESS 1972b) The decay of the esterase activity after contact activation was studied at various temperatures (fig 2) The maximum esterase activity developed later at 15° and 0° than at 37° More striking, however, was the very slow decay of the esterase activity at 0° This might have been caused by suppression of the activity of C$\bar{1}$-inactivator and the formation predominantly of the esterase active complex between plasma kallikrein and α_2-macroglobulin However, heating at 37° after 60 minutes' incubation of the kaolin plasma mixture induced inhibition, thus indicating that the esterase was still subject to inhibition by both C$\bar{1}$-inactivator and α_2-macroglobulin

The rate of the reaction between the activated prekallikrein and the inhibitors in plasma was evaluated at 25° and 0° with regard to the kinin-liberating effect (fig 3) At 0° the liberation of kinin from heated plasma is linear with the concentration of plasma kallikrein up to 0 7 µg bradykinin equivalent per ml of the substrate (VENNERØD & LAAKE, unpublished results), and the kinin release can therefore be taken to represent the activity of kininogenase In the experiment cited in fig 3 the half-times of the kininogenase activity were 1 2 and 120 minutes at 25° and 0°, respectively On the

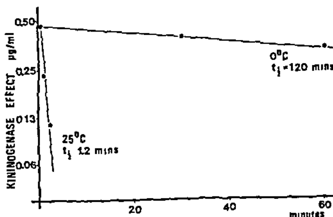


Fig 3 Inhibition of the kininogenase activity of plasma kallikrein at 25° and 0° Reference plasma was activated at 0° with kaolin for 10 minutes and 0 06 ml of the plasma kaolin mixture was incubated for 30 minutes at 0° with 0 4 ml of the purified preparation of prekallikrein The reaction mixture which contained activated plasma kallikrein was then incubated at 25° and 0° with an equal volume of EDTA 2Na treated reference plasma to supply inhibitors, and Polybrene (Aldrich Chem Co Inc Milwaukee, Wisc U S A) was added to a final concentration of 0 1 mg/ml to arrest further activation of prekallikrein At intervals, 0 1 ml of the mixture was added to 0 5 ml 60° heated kininogen substrate at 0° and the kinin release during 15 minutes was determined

other hand, the kinin-liberating effect of the preparation was 6 6 times higher at 25° than at 0°

4 Estimation of total kininogen and kininogen fractions

Plasma kallikrein and glandular kallikrein react with different fractions of kininogen in plasma (BRISEID *et al* 1970, 1973) Experiments were undertaken to estimate the total kininogen concentration and the kinin release obtained with plasma kallikrein (substrate 1) and hog pancreas kallikrein (substrate 2 essentially), respectively (table 3) The total kininogen concentration in plasma was found to be significantly higher during pregnancy and treatment with oestrogenic oral contraceptives than in untreated, non pregnant subjects The increase was confined to the kininogen fraction activated by hog pancreas kallikrein Within each group the total concentration of kininogen was somewhat higher in the cold activation positive than in the cold activation negative plasmas, and a similar trend was seen in the fractions activated by the preparation of plasma kallikrein and hog pancreas kallikrein

Discussion

For the estimation of prekallikrein in plasma the determination of the maximum activity towards arginine esters after activation with kaolin is probably a valuable method Our observations that plasma prekallikrein was

Table 3

Total kininogen concentration and concentration of kininogen fractions

Group	Pregnancy		Contraception		Untreated	
Cold activation	Positive*	Negative**	Positive*	Negative**	Positive**	Negative*
Total kininogen	4 39 ± 0.59	4 10	4 56 ± 0 86	4 30	3 92	3 29 ± 0 35
Substrate 1	0 82 ± 0 06	0 97	1 07 ± 0 16	0 91	0 92	0 96 ± 0 12
Substrate 2	4 43 ± 0 60	2 96	3 90 ± 0 41	3 20	2 21	2 37 ± 0 26

The total kininogen concentration (BRISEID *et al* 1967) was estimated by incubation of 1 0 ml EDTA 2Na treated plasma for 24 hours with 0 5 ml acetone and 3 5 ml 0 15 M sodium chloride and subsequent determination of the kinin The concentration of substrate 1 (BRISEID *et al* 1970) was determined by incubation of the EDTA 2Na treated plasma with an equal volume of EDTA 2Na treated preparation of plasma kallikrein for 10 minutes at 37° The concentration of substrate 2 was essentially determined by incubation for 30 minutes at 37° with hog pancreas kallikrein (padutin®) final concentration 3 2 biological units per ml EDTA 2Na treated plasma (BIELTVEDT & BRISEID 1967) The results are given as µg bradykinin equivalent per ml plasma

* The means of five observations with 95 % confidence interval

** The means of two observations

increased in pregnancy and during treatment with oestrogenic oral contraceptives, are in good agreement with those of COLMAN *et al* (1969b) The method does not take into account variations in the concentration of factor XII However, previous studies by GJØNNAESS (1972a) disclosed no differences between the cold activation positive and negative plasmas with regard to factor XII Furthermore, it has not been settled whether the increased esterase activity after kaolin activation represents increased concentration of the proenzyme or merely increased esterase activity of the kallikrein molecule At present, it seems most fruitful to consider the first possibility

The concentration of prekallikrein was higher in the cold activation positive plasmas than in the negative plasmas within each group The agreement however, was incomplete, since the prekallikrein concentration in the cold activation positive plasmas from women on oral contraceptives was similar to that in the cold activation negative plasmas from pregnant women (table 1) *Furthermore, purified preparations of plasma prekallikrein are* stable (WUEPPER & COCHRANE 1971), and the proenzyme has not been reported to have esterase or proteinase activity However, assuming that factor XII is activated in the cold and gives spontaneous activation of the kallikrein-kinin system as suggested by ARMSTRONG & DA SILVA (1970), then *an increased concentration of prekallikrein in the positive plasmas may lead* to an increased formation of kallikrein if the K_M for the effect of activated factor XII on prekallikrein is in the range of the physiological concentration of prekallikrein in plasma

The activities and the concentrations of inhibitors of proteinases in plasma are influenced by oestrogenic hormones (BODMAN 1958, LAURELL *et al* 1968, FAGERHOL & ABILDGAARD 1970) Both $\overline{C1}$-inactivator and α_2-macroglobulin are potent inhibitors of plasma kallikrein (KAGEN 1964, HARPEL 1970), and variations in the inhibitor activity might be related to the occurrence of the cold activation of factor VII However, neither the kinetics of the inhibition of the kaolin activated esterase at 37° nor the results of the immunological determinations of various proteinase inhibitors in plasma substantiated such a view (table 2) Furthermore, at 0° the reaction between the kininogenase and the inhibitors in plasma was markedly suppressed (figs 2 and 3) whereas the kininogenase was still quite active towards kininogen If *plasma kallikrein can activate factor VII, then the marked temperature* dependence of the inhibition of the kininogenase activity may explain why the activation takes place at low temperature only The temperature dependence of the kallikrein inhibitors is presumably similar in all plasmas, and the observed temperature dependence does *not* explain why only some plasmas show spontaneous cold activation of factor VII Whether such marked temperature dependence also applies to the inhibition of other proteinases is largely unknown, NOSSEL & NIEMETZ (1965), however, ob-

served slow inhibition of the contact product of blood coagulation at 4°, while the activity of C$\bar{1}$ inactivator towards C$\bar{1}$ is low at 10° (LEPOW *et al* 1958)

The inhibitory effect of plasma on kininogenase activity represents an obstacle for studies on the kinin liberating effects of the kallikreins on kininogen Various methods, such as treatment of plasma with acid (HORTON 1958) and acetone (BRISEID *et al* 1968), have been reported to suppress the effect of the inhibitors As kinin was still released at 0° and the effects of the inhibitors were strongly suppressed at this temperature, cooling may represent a valuable tool in such studies

Using the method of DINIZ *et al* (1961), PERITI & GASPARRI (1966) observed an increased concentration of kininogen in plasma during pregnancy Our results (table 3) support their findings and disclosed similar changes during treatment with oral contraceptives The kininogen increase was mainly confined to the kininogen substrate 2 of BRISEID *et al* (1970, 1973) Our results were not correlated with the concentration of protein in the plasmas, the insignificant differences in the kininogen fraction 1 (table 3), however, indicated that the relative concentration of the different kininogen fractions were actually altered during pregnancy and treatment with oral contraceptives Isolated fractions of human kininogens have been reported to exert neither esterase nor proteinase activity, and we do not feel that the higher concentration of kininogen in the cold activation positive plasmas can account for the activation of factor VII

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cholinergic mechanisms in the brain However, to be able to study the central effects of oxotremorine, the peripheral muscarinic effects had to be blocked with methylatropine The above conclusion thus implies that the brain concentration of methylatropine in the newborn dog is not that much higher than in the adult dog and that it can block the central effects of oxotremorine However, there are some reasons for suspecting that this is the case Firstly, methylatropine is eliminated from the ventricular cerebrospinal fluid (CSF) partly by an active transport mechanism in the adult dog (AQUILONIUS & WINBLADH 1972) Secondly, other quaternary amines such as tetraethylammonium (TEA) are excreted in the kidney partly by tubular secretion (RENNICK *et al* 1954) Such active transport mechanisms are not developed at birth in the dog at least not with regard to paramino hippuric acid (PAH) (HOOK *et al* 1970, HOLLOWAY & CASSIN 1972a) Thirdly the general concept is that the blood brain barrier is more permeable in immature animals (cf DOBBING 1968) The present study was thus under taken to determine to what extent immaturity influences the fate of methyl atropine in the dog

Methods

15 beagle puppies of both sexes from 7 litters were used at the following age inter vals newborn (1 5 days) three weeks (18–25 days) 6 weeks (40–50 days) and 3 months (85 100 days) The experimental procedure was the same as described earlier (WINBLADH 1973a) and special care was taken to avoid hypothermia in the newborn group The labelled methylatropine was synthesized from atrop ne generally tritiated 434 mci/mmol (Radiochemical Centre Amersham England) and checked for radiochemical purity as described elsewhere (WINBLADH 1972a) The labelled drug with the necessary amount of carrier methylatropine nitrate was injected subcutaneously into the neck or intravenously in distilled water in a volume of 1 ml/kg to the two youngest age groups and 0 1 ml/kg to the older dogs The injected radioactivity varied between 50 and 200 µci/kg Blood and urine sampling was done via catheters inserted before the experiments with the animals under halothane anaesthesia Determination of tissue con centrations of radioactivity was done by liquid scintillation counting after oxygen combustion while the radioactivity in the blood urine CSF and bile was determined after direct mixing with the scintillation solvent The different parts of the brain from the hemispheres were obtained by cutting out a piece of tissue stretching from the lateral ventricle to the parietal cortex and then dividing it by three equidistant transverse sections Invest gation of metabolites in the urine was done by paper chromatography (Whatman no 1 butanol acetic acid (5 1) satured with water) and h gh voltage electrophoresis (Whatman no 1 borate buffer 0 5 M pH 10 20 V/cm 2 hrs) Calculation of half life of total plasma radioactivity was made by fitting a line according to the least square method to all values from one group on parts of the curve [illegible] logarithmic plot with the aid of a FOCAL [illegible] ll the above methods have been described

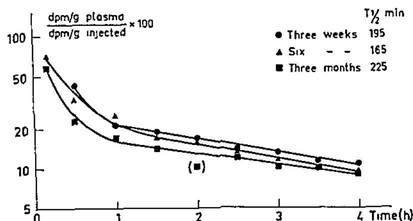


Fig 1 Plasma concentrations of radioactivity after intravenous injection of 0 5 mg/kg of methylatropine in three dogs of different ages Each curve represents one dog T ½ = plasma half life of radioactivity for the linear curve part

Results

Plasma concentrations

The plasma concentration of radioactivity after a single intravenous dose of 0 5 mg/kg was below that of a uniform concentration within 10 min During the first hour there was a rapid non-linear decrease in concentration in the semilogarithmic plot (fig 1) From 1 hr after injection the disappearence

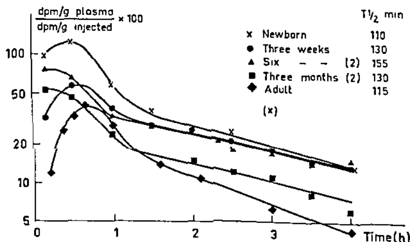


Fig 2 Plasma concentrations of radioactivity after subcutaneous injection of 0 5 mg/kg of methylatropine to dogs of different ages For six weeks and 3 months old dogs curves represent mean from two dogs other curves represent one dog T ½ = plasma half life of radioactivity for the linear curve part

curve is linear and there are no significant differences in the concentrations or half lives (165–224 min, fig 1) between the investigated age groups, i e 3 and 6 weeks and 3 months old animals After subcutaneous injection of 0 5 mg/kg a peak concentration of radioactivity was reached within 40 minutes The peak concentration was three times higher in the newborn dog than in the adult, while the concentrations in the other groups fell inbetween After the concentration peak there was a *non linear decrease for* about one hour in the semi-logarithmic plot (fig 2) After this phase the dissappearence curve is linear as in the intravenous injections Half life of plasma radioactivity during this phase was slightly shorter for the newborn and the adult dog than for the other age groups (110–115 min versus 130–155 min, fig 2) The *plasma concentration at 4 hrs after injection in the* 3 months old and in the adult animal was ½ and ⅓ respectively that of the younger age groups However, in the experiments where tissue concentrations were investigated no consistent differences in plasma concentrations between newborn and adult animals were found at 2 hrs after injection (table 2)

Urinary excretion

The urinary excretion of radioactivity was between 40 and 70 per cent of the injected amount at 4 hrs (table 1) Between 60 and 100 per cent was in the form of the unchanged drug according to the electrophoretic studies, except in the 3 months old dog where only 25 per cent was re covered as methylatropine There were no consistent differences in excretion

Table 1

Urinary excretion 4 hrs after injection

	Route and dose	$\frac{\text{Excreted radioactivity}}{\text{Injected radioactivity}}$ 100	Per cent methylatropine**	Plasma concentration***
Newborn	s c 0 5	52	66 50 – 56 75	13
3 weeks	i v 0 5	44	50	11
	s c 0 5	39	70	13
6 weeks	i v 0 5	45		10
	s c 0 5	67 63	79 100	21 10
	s c 0 1	40 45	– 100	11 –
		47	76	10
3 months	s c 0 5	45 72	– 25	8 7
Adult*	s c 0 1	55	83	5

* Data from ALBANUS *et al* 1969

** Per cent of total activity recovered from the electropherograms

*** Given as $\frac{\text{d p m /g plasma}}{\text{d p m /g injected}} \times 100$

Table 2

Tissue concentration* of radioactivity 2 and 4 hrs after subcutaneous injection of methylatropine

	2 hrs		4 hrs			
Age group	newborn	adult**	newborn		6 weeks	
Dose mg/kg	0 1	0 1	0 1	0 5	0 1	0 5
Kidney	–	–	–	85 34	126	107
Liver	207	393	360	322 392	662	551
Bile	236	3400	–	2700 3500	22000	10500
Plasma	15	14	9	13 –	10	11

* Given as $\frac{\text{d p m /g tissue}}{\text{d p m /g injected}} \times 100$ Each value represents one animal

** Data from ALBANUS *et al* 1969

rate or fraction of metabolites between the different age groups Nor was there any correlation between the excreted amount in 4 hrs, the fraction of metabolites in the urine collected between 2 and 4 hrs, and the plasma concentration at 4 hrs after injection (table 1) The high voltage electrophoretic procedure gave a more efficient separation of the metabolites than did the chromatographic separation The pattern of metabolites was qualitatively the same as that reported for adult dogs (ALBANUS *et al* 1969), except in the three months old dog which showed only 25 per cent unchanged drug in the urine This animal had a urinary peak of radioactivity in the chromatogram with a lower Rf-value, 0 47, in addition to the two peaks usually found, 0 55–0 57 and 0 66–0 72 (methylatropine) This peak represented about 15 per cent of the recovered activity

Tissue concentrations of radioactivity

The concentrations of radioactivity in the kidney and liver at two and four hrs after injection were usually more than five times higher than that of the plasma All values are shown in table 2 Two hrs after subcutaneous injection of 0 1 mg/kg of methylatropine the concentration of radioactivity in the liver was almost twice as high in the adult dog as in the newborn The bile concentration was about 15 times higher, while the plasma concentrations were the same The differences between newborn and six weeks

Table 3

Brain concentrations of radioactivity* 2 and 4 hrs after subcutaneous injection of 0 5 mg/kg

	2 hrs		4 hrs	
Age group	Newborn	Adult***	Newborn	6 weeks
Hemisphere**				
Slice I	11 1	10 3	1 2	2 9
Slice II	14 2	13	1 5	4 3
Slice III	16 2	11	2 9	3 3
Slice IV	15 0	18 8	4 2	5 9
Thalamus	–	9 4	1 1	–
Cerebellum	–	7 9	5 9	–
CSF	2 4	2 8	0 9	1 9
Plasma	15	15	13	11 2

* Concentrations given as $\frac{\text{d p m /g tissue}}{\text{d p m /g injected}} \times 100$

(10 corresponds to 0 05 μg/g of the unchanged drug)

** Slice I nearest to the lateral ventricle slice IV parietal cortex

*** Data from ALBANUS & WINBLADH 1969

old dogs at four hrs after injection showed a similar pattern i e the older dogs had higher kidney, liver and bile concentrations than the newborn, but they had about the same plasma concentrations

In the brain hemispheres there appeared to be a fall in the concentration of radioactivity from the parietal cortex towards the lateral ventricles (table 3) The concentration in the cortical slice was 1 5–3 5 times larger than that in the slice nearest to the ventricle This slice in its turn had a 1 3–4 5 times larger concentration than the CSF collected in the cisterna magna Apart from these findings there were no significant differences in concentrations either between the different brain regions or between the different age groups

Discussion

In general there appears to me quite small differences in distribution, meta bolism, and excretion of methylatropine between dogs of different ages There were no large differences in plasma half life or concentrations of radioactivity between newborn and adult dogs in contrast to the finding with atropine After both subcutaneous and intravenous administration of this drug adult

dogs had a shorter half life and lower plasma concentrations than newborn dogs (WINBLADH 1973a) Further, after atropine the rate of urinary excretion of radioactivity was only half of the adult rate in newborn and six weeks old dogs (WINBLADH 1973a) It has been shown that atropine is partly excreted in the urine via tubular secretion (ALBANUS *et al* 1968) and it was suggested that the slower excretion of this drug in the newborn is due to an imunaturity in the tubular transport mechanism (WINBLADH 1973a) as shown for para aminohippuric acid (HOOK *et al* 1970) It appears likely that methylatropine is also excreted partly by tubular secretion, since this drug is excreted somewhat faster than atropine in the adult animals at similar plasma concentrations and with about the same fraction of metabolites in the urine The reason why no difference in the rate of excretion of methylatropine was found between the newborn, six weeks old and adult dogs is not evident. One possible explanation would be that two transport systems are involved and that the transport of methylatropine develops earlier Another explanation is that the more lipid soluble atropine rediffuses from the tubules to plasma to a greater extent than the less lipid soluble methylatropine, thus exceeding the transport capacity of the tubules in the young dogs There is a progressive increase in the accumulation of TEA in renal slices from dogs with increasing age (RENNICK *et al* 1961) At least in the liver it appears as the secretory mechanisms for methylatropine and for its metabolites are not yet developed in the newborn dog, since bile concentrations of radioactivity are considerably lower in this age group than in the adult This difference is not likely to be due to differences in the rate of the hepatic degradation of the drug because there is no difference in the fraction of the metabolites in the urine between the different age groups

There were no differences in CSF concentration between newborn and older dogs The rate of accumulation of methylatropine in the isolated choroid plexa was approximately the same in the tissue of dogs of different ages (WINBLADH 1972b), while an increase with age has been reported for PAH (HOLLOWAY & CASSIN 1972b) This increase in choroid plexus transport of PAH *in vitro* was reflected in a lower CSF clearence of the drug in newborn dogs than in the adult (HOLLOWAY & CASSIN 1972a) However, the CSF clearence of methylatropine has not been studied in dogs of different ages

As described above there was a concentration gradient of radioactivity within the hemispheres with the lowest concentration towards the lateral ventricle One possible explanation for this finding would be a diffusion of the drug towards the lateral ventricle due to a constant removal of the drug from the ventricular CSF by CSF bulk flow and active removal via the choroid plexa However, there are other factors which might be responsible for this distribution picture (WINBLADH 1973a & 1972b)

There were no significant differences however in brain concentrations or concentration gradients between the dogs of the different ages studied Thus, the lack of tremorogenic action of oxotremorine in the newborn puppies does not depend on a high brain concentration of methylatropine

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Organ Bath with Controlled P_{CO_2}

By

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Abstract Two simple arrangements are described by means of which the P_{CO_2} and P_{O_2} of physiological salt solutions placed in open vessels can easily be adjusted and maintained at known and constant levels during biological experiments on immersed living tissue The characteristic feature of the proposed arrangements is that a certain fraction of the area of the free surface of the fluid under standardized stirring conditions is exposed to an atmosphere with known and constant concentrations of CO_2 and O_2 The constancy of the P_{CO_2} and the P_{O_2} in the fluid is practically uninfluenced by the additional stirring caused by the biological experiments for which the bath is used

Key words Carbon dioxide environment controlled – surviving tissue

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The behaviour of isolated living tissues is greatly influenced by environmental conditions Therefore, in most experiments with surviving tissue, a constant and well-defined environment is indispensable (AHLGREN 1930, ELLIS 1969)

For many experimental purposes this is achieved by immersion of the tissue in a suitable solution of known and constant composition and temperature

The concentration of the non volatile substances of this bathing fluid will remain practically constant during the experiment if the volume of the bathing fluid is made large enough as compared with the bulk of tissue, and the temperature of the bath can easily be controlled by well known measures However, the precise control of the concentration of dissolved volatile substances may require special attention in biological experiments in which the experimental procedure necessitates exposure of the surface of the bathing fluid to the atmospheric air

Because of the influence of the concentration of dissolved CO_2 upon the pH, it may be of paramount importance to control the P_{CO_2}, whereas it is generally sufficient merely to keep the P_{O_2} above a certain critical level

In most biological work with isolated tissue the concentration of other dissolved gases is without significance

In experiments of short duration it may be sufficient just to provide an initial equilibrium between the bathing fluid and a suitable mixture of gases However, for experiments of longer duration this is not enough It should be realized that bubbling of a fluid placed in an open vessel with air containing known concentrations of CO_2 and O_2 is unsuitable for providing diffusion equilibrium between the air used for bubbling and the fluid The resulting concentration of CO_2 (and O_2) is not constant and often approaches equilibrium rather with the air of the room than with the air mixture used for bubbling (KLINGELHOFFER 1929) The main reason is that the total surface of the simultaneously ascending air bubbles generally constitutes only a small fraction of the free surface of the fluid which is exposed to the air of the room

A description is given below of two arrangements suitable for the establishment and maintenance of constant P_{CO_2} and P_{O_2} in fluids, such as physiological salt solutions placed in open vessels with the surfaces exposed to the air of the room The steady state P_{CO_2} and P_{O_2} of the fluids is, within wide limits, unaffected by varying intensities of the stirring of the fluids

Preliminary experiments

The procedures were worked out with organ baths contained in box shaped glass vessels of different shapes and sizes In most experiments vessels measuring about 21 × 34 × 25 cm (height) were used The air exposed surface of the fluid was about 720 cm² During the experiments the vessels were filled up to 4 cm below the upper rim thus giving a volume of bathing fluid of about 15 litres

In all experiments described below, the bathing fluids were stirred by a rotating circular disc mounted concentrically at the end of the shaft of an electric a c motor making about 1400 revolutions per minute In most experiments the disc was immersed 5 cm into the fluid with the shaft placed vertically Different and reproducible intensities of stirring were obtained without changing the speed of the rotating shaft of the stirrer simply by the selection of discs of different diameters (up to 6 cm) The thickness of the discs was 3 mm in all experiments

The first experiments were carried out to determine the rate of the exchange of CO_2 through the free surface of water placed in the above mentioned glass vessels under different conditions In these experiments the bathing fluid was distilled water slightly acidified with citric acid (1 0 mM) An initial CO_2 concentration of about 5 mM in the fluid was provided by the arrangement shown in fig 2 and the concentration of CO_2 dissolved in the fluid was determined at suitable time intervals by a method based on measurements of the electrical conductivity of a solution of $Sr(OH)_2$ before and after the absorption of the quantity of CO_2 contained in a small sample of the above mentioned fluid (HOLM JENSEN 1960 1963 & 1965) The half times of the processes of CO_2 elimination were read from the linear plots on semilogarithmic paper of the CO_2 concentration against time

Experiments which were carried out with 3 or 4 baths simultaneously revealed that at room temperature the half life of dissolved CO_2 with a stirring disc of a

diameter of 4 cm, which gave a fairly vigorous stirring was about 3.5 hrs when the shaft of the stirrer was placed in a corner 6 cm from each of the adjacent glass walls With this position of the stirring shaft furnished with the 2.5 cm disc, the half time was about 5 5 hrs and with the shaft of the motor alone without any stirring disc mounted the half life was about 13 hrs Experiment at 37° with the 4 cm stirring disc placed in a corner as described above showed that CO_2 escaped with a half life of about 2 4 hrs

In experiments with a fan blowing air on the surface of the fluids it was found that a moderately increased stirring of the air of the room did not significantly influence the half time of the CO_2 elimination

First arrangement

The first of the proposed arrangements, fig 1, is intended for experiments in which only half of the surface of the bathing fluid needs to be freely accessible for the procedures required by the biological experiments, the

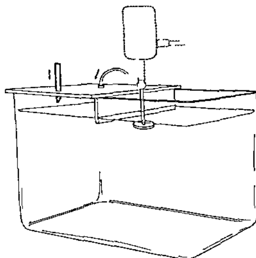


Fig 1 Organ bath with half of the surface of the fluid exposed in a separate air chamber to (a current of) carboxygen or some other air mixture with a suitable concentration of CO_2. The roof and the vertical partition plate of this compartment are made of Perspex cemented airtight together and to the upper rim of the glass vessel The lower rim of the vertical partition plate is immersed 1 to 2 cm below the surface of the fluid The roof is provided with a nozzle for the inlet tubing and an outlet tube with an internal diameter of 0 6 cm, which is the minimum diameter required to avoid obturation of the tube by droplets of condensed water With this diameter, the length must be at least 6 cm to avoid admixture of atmospheric air The vertical shaft of the stirring motor penetrates the surface near the middle of the bath close to the Perspex partition plate The shaft making about 1400 revolutions per minute is provided with a circular stirring disc – diameter, for most purposes 4 cm – which is immersed 4 to 5 cm below the surface A suitable flow rate of carboxygen is about 7 ml/min per 100 cm² free surface of the bath

other half of the surface being exposed to an atmosphere in a closed compartment through which carboxygen Ph Nord (a mixture of 4 % CO_2 v/v and 96 % O_2 v/v) is passed

Experiments revealed that with a flow rate of carboxygen of about 50 ml/min per 700 cm² fluid surface the CO_2 concentration of the fluid of the bath approached a steady state corresponding to equilibrium with an air mixture with a CO_2 concentration of about 45 % of that of the carboxygen used for the experiment In this arrangement with the partition plate dipped 2 cm below the surface and with the 4 cm stirring disc and slightly acidified solutions, the half time of the approach to the steady state was about 3 hrs at 37° The steady state concentration of CO_2 in the fluid was not influenced measurably by variations of the stirring intensity caused by changes of the stirring disc diameters from 2 5 to 6 cm The steady-state

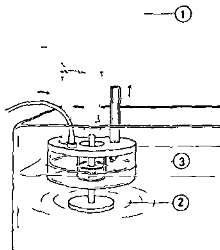
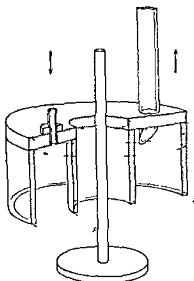


Fig 2a Unit comprising motor 1 with shaft and circular stirring disc 2 and air chamber 3, for the establishment of contact between CO_2 and a constant portion of the surface of the fluid under well defined stirring conditions The air chamber which is firmly fixed to the shell of the motor is made of Perspex It consists of two concentric tubes length 3–4 cm cemented to a roof, leaving the aperture of the inner tube uncovered for the passage of the stirring shaft The roof is provided with an inlet nozzle and an outlet tube for the CO_2 gas (dimensions as in fig 1) When in use the lower rim of this air bell is immersed 1–2 cm below the surface of the fluid The distance between the lower rim of the chamber and the stirring disc is 3 cm A suitable flow rate of the CO_2 gas is about 2 ml/min per 100 cm² free surface of the bath



F g 2b Sect onal v ew of the CO_2 chamber for an organ bath w th an a r exposed surface of 720 cm² An a r chamber w th an outer d ameter of the inner tube of 3 0 cm and an inner d ameter of the outer tube of 5 25 cm g v ng a bottom area of the CO_2 chamber of 14 6 cm² proved very su table for b olog cal work The steady state of the bath ng flu d corresponds to equ l br um w th an atmosphere contain ng about 4.5 % CO_2

concentration of O_2 which was determined in a few experiments corresponded to equilibrium with air containing about 55 % of oxygen

If the above arrangement is used with physiological salt solutions containing 10 meq /l of bicarbonate which is about 40 % of the bicarbonate concentration of blood plasma the pH of the solution will be suitable for most experiments on surviving tissues from homoiothermic animals while a precise adjustment of the pH can conveniently be made by changing the concentration of bicarbonate

Second arrangement

The second arrangement fig 2 is intended for experiments in which it is important that nearly the whole of the free surface of the bath is accessible for the biological experiments The principal feature of this arrangement is that a small fract on of the free surface of the bath is exposed to air in a chamber through which pure carbon dioxide is passed Stirring of the fluid is carried out with the above mentioned discs placed with their centres in a fixed position relative to the CO_2-filled air compartment

With the arrangement shown in fig 2 the steady state concentration of CO_2 dissolved in the fluid was determined to be between 2 0 and 2 4 times

the level expected if the CO_2 saturation was proportional to the fraction of the fluid surface exposed to the CO_2 atmosphere

Experiments revealed that doubling and halving the area exposed to the CO_2 atmosphere by changing the outer diameters of the CO_2 chambers did not change the above factor of proportionality, and that changing the stirring intensity by exchanging discs beween diameters 2 5 and 6 cm did not influence the steady-state concentration

The influence of additional stirring elsewhere in the bath proved to be negligible when the diameter of the additional stirring disc did not exceed one half of the diameter of the disc of the CO_2 chamber

The steady-state equilibrium was found to be practically uninfluenced by reduction of the CO_2 flow down to a minimum of about 2 ml/min per 100 cm² free surface of the bath

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The Rotacone: A New Apparatus for Measuring Motor Coordination in Mice

By

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(Received November 6 1972, Accepted March 22, 1973)

Abstract The rotacone is a modified rotarod found suitable for the measurement of drug induced changes in motor coordination On this apparatus mice are forced to walk against increasing surface speed until they drop off the cone As the test is quick the animals are not exhausted, and there is no problem with mice taking "free rides" Saline treated mice showed a constant performance level in 4 immediately successive trials whereas animals under the influence of drugs especially chlordiazepoxide, showed an increase in performance in the 4 trials After chlorpromazine, meprobamate and chlordiazepoxide there is a graded response with a normal distribution of scores Pentobarbital gave a response of the all or non type

Key words Behaviour, animal – chlordiazepoxide – meprobamate – barbiturates – phenothiazines

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Various techniques used for the measurement of motor coordination were reviewed by KINNARD & WATZMAN in 1966 The first horizontal rotating rod, the rotarod, was described by DUNHAM & MIYA 1957 Mice were trained until they were able to remain on the rod for one minute Disturbance in motor coordination was indicated by the inability of the animal to remain on the rotarod for the one minute test period

WATZMAN & BARRY 1968 used a modified rotarod test They increased the rotation speed every 30 sec, and measured the time spent on the apparatus until the animals fell off In this way they were able to measure both decrease and increase in performances caused by drugs A similar principle was used by JONES & ROBERTS 1968 in the accelerating rotarod With both methods graded responses were obtained The test time to obtain a full performance in the non drugged state was approximately 2 and 4 min, respectively The present work describes another modification of the rotarod On the rotacone the animals are forced to walk against increasing surface speed The test is quick as it takes about 20 sec

to test a mouse, and due to the short test time exhaustion plays no role in the measured performance

Another problem which has been overcome by this method is the problem of mice taking ' free rides", i e the animals cling to the rod without walking This behaviour enables some animals to stay on the rod even when motor coordination is impaired by chlorpromazine (WATZMAN *et al* 1967) On the *rotating cone such a clinging does not tend to overestimate the performance* The rotacone was developed by E JACOBSEN (Danish Patent No 117598 1970)

Materials and Methods

Animals

Male albino mice (24 30 g) of the NMRI strain from Møllegaard s Breeding Laboratories were used

Equipment

The rotacone consists of a cone with a suitably rough surface A wall wound in a spiral on the surface of the cone forces the animals to move along the cone against increasing diameter and with this an increasing surface speed resulting in a maximum speed which is 3 times the initial one Through a gearbox the cone is coupled to a motor This makes it possible to use different speeds of rotation ranging from 10 to 40 r p m *When the mice are no longer able to maintain equilibrium on the rotacone* they fall into one of ten boxes placed under it The boxes are numbered 0 to 9 from the right to the left The performance of a mouse is defined as the box number in which it falls Mice falling in box No 9 show the maximal performance The rotacone is seen in fig 1

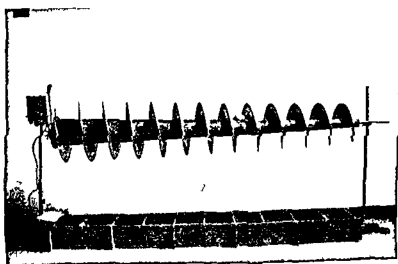


Fig 1 The rotacone with ten boxes placed underneath Length of the cone 1250 mm Diameters 35 mm to 115 mm

Training

Generally the training takes 3 days On the first day the mice are trained at a low speed 18 r p m on the following day at the test speed 30 r p m After 2 or 3 runs on the third day the mice reach a performance level, constant both in successive tests and from day to day The individual normal performance was measured immediately before the injection of the drug

Measuring performance

The effect of a drug is expressed as the activity ratio (AR), which is the performance after injection of the drug divided by the normal performance for the experimental animal Mice having normal performances corresponding to box 4 and less were not used in the experiments

Results

Performance of untreated trained mice

The test speed of 30 r p m gave normal performances with most untreated trained animals falling into boxes number 5, 6, 7 and 8 and only a few into boxes number 4 and 9 At a slower speed too many animals were able to run all the way A faster speed could be used to test improvements in performances The distribution of performances for 30 r p m is seen in fig. 2 Each performance is the mean performance of a single mouse in 4 immediately successive trials, i e the mouse is replaced on the rotacone immediately after the previous trial

Fig 3 shows that untreated control mice and saline injected mice in 4 immediately successive trials have constant performance levels, and no sign

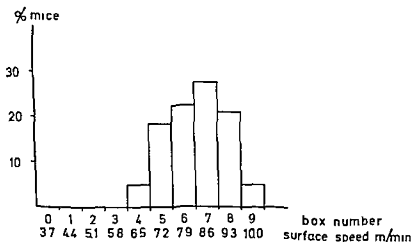


Fig 2 Distribution of normal performances on the rotacone of 200 trained mice (30 r p m) Each performance is the mean of 4 immediately successive trials for a single mouse

The responses in fig 5 give the results for compounds tested For chlor promazine, meprobamate and chlordiazepoxide there are graded responses with normal distribution of scores Pentobarbital sodium (mebumalum NFN) dissolved in 0 9 % saline and given intraperitoneally half an hour before the test gave a response of the all or non type After 15 mg/kg no decrease in performance was seen, but after 22 5 and 30 mg/kg 3 and 12 mice, respectively, out of 16 had an AR of less than 0 2 while the remainder showed no difference from the saline treated control tests

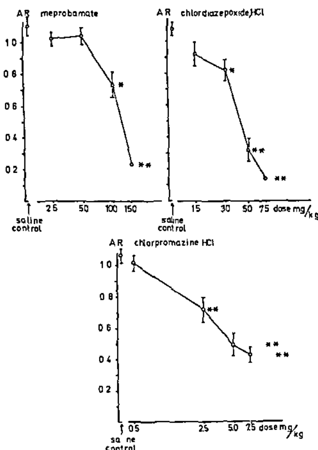


Fig 5 The AR as a function of varying doses of meprobamate chlorpromazine HCl and chlordiazepoxide HCl Administration of drugs and saline as in fig 3 The AR is the highest performance in 4 immediately successive trials ½ hour after the injection of the drug divided by the mean performance in 4 immediately successive trials just before injection of the drug for a single mouse The mice were kept at 31° from the injection to test Each point is the mean of 16 mice ± S E M (vertical lines)

* $P < 0.01$ compared with saline control

* * $P < 0.001$ compared with saline control

(student s t test)

Discussion

The rotacone was found suitable for the measurement of drug induced disturbances in motor coordination, giving a sensitivity which seems to be equal to the above mentioned accelerating rotarod (JONES & ROBERTS 1968) For chlordiazepoxide, however, the sensitivity seems to be decreased in the present apparatus as compared to the conventional rotarod (SOFIA 1969) This is probably due to the increase in performance in the 4 immediately successive trials especially found after chlordiazepoxide in this study

The reason for this increase in performance is not clear, but it cannot be due to a decrease in the blood level of the compound and its metabolites during the test period, since it takes less than 80 sec to carry out the 4 trials As seen from DENCKER CHRISTENSEN 1973, the level of the only measurable compound in the plasma ½ hour after injection of chlordiazepoxide, HCl 50 mg/kg, the metabolite D M (7-chloro-2-amino-5 phenyl 3H-1,4-benzodiazepine-4 oxide) is rather constant

The phenomenon might be dependent on learning or adaption to the test situation, and is under further investigation

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Tolerance Development with Chlordiazepoxide in Relation to the Plasma Levels of the Parent Compound and its Main Metabolites in Mice

By

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Abstract A single pretreatment with chlordiazepoxide, HCl (50 or 165 mg/kg) to male mice produced tolerance to the compound 24 hours later as measured by the performance on a modified rotarod (the rotacone) The plasma levels of chlordiazepoxide and the major metabolites 7 chloro-2 amino-5 phenyl 3H 1 4 benzodiazepine-4 oxide (D M) and 7-chloro 1 3 dihydro-5 phenyl 2H 1,4-benzodiazepine-2 one-4 oxide (lactam") were determined in mice acutely and repeatedly treated with chlordiazepoxide Although a single pretreatment of chlordiazepoxide HCl increases the rate of metabolism other factors must be involved to explain the tolerance developed. Mice treated twice with chlordiazepoxide HCl 165 mg/kg had significantly higher plasma levels of the compound and its metabolites 10 and 30 minutes after the second injection (given 24 hours after the first injection) as compared with mice receiving 50 mg/kg chlordiazepoxide HCl acutely, while the performance on the rotacone was similar in the two groups Chlordiazepoxide had the most potent effect in the rotacone test D M and 'lactam' were about equipotent

Key words Behaviour animal – chlordiazepoxide – drug tolerance

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Chlordiazepoxide (clopoxidum NFN) has been reported to cause tolerance after repeated treatment of mice and rats HOOGLAND *et al* (1966) showed that treatment for 5 days with 100 mg/kg chlordiazepoxide to rats produced tolerance to the skeletal muscle relaxant effects of the compound These rats showed increased rates of tissue disappearance and excretion of ^{14}C-labelled chlordiazepoxide as compared with non-tolerant rats due to stimulation of the hepatic microsomal enzymes responsible for drug metabolism, suggesting that this stimulation was involved in the mechanism of tolerance development

GOLDBERG *et al* (1967) reported that tolerance to many effects was developed after chronic administration of chlordiazepoxide to mice and rats

Evidence was found suggesting that increased metabolism participated in the underlying mechanism

The aim of the present study was to examine whether the increase in metabolism and excretion could explain the tolerance seen after repeated treatment with chlordiazepoxide to mice This was done by comparing the performance on the rotacone (DENCKER CHRISTENSEN 1973) with the plasma levels of chlordiazepoxide and its major metabolites, 7-chloro 2 amino 5-phenyl-3H-1,4-benzodiazepine-4-oxide (D M) and 7-chloro-1,3-dihydro-5-phenyl 2H-1,4 benzodiazepine-2-one-4-oxide ("lactam") shown in fig 1 (COUTINHO *et al* 1969)

Materials and methods

Animals

Male albino mice (24–30 g) of the NMRI strain from Møllegaard Breeding Laboratories were used

Measuring drug effect

Motor coordination was measured using the rotacone, a modified rotarod (E JACOBSEN Danish Patent No 117598 1970, DENCKER CHRISTENSEN 1973) The activity ratio (AR) is the highest test score in 4 immediately successive trials 10 30 or 60 min after injection of the drug divided by the normal performance for a single trained mouse A low AR indicates a poor performance

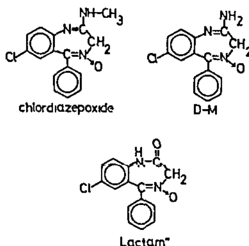


Fig 1 Chlordiazepoxide and its main metabolites in mice

Chlordiazepoxide	7 chloro-2 methylamino-5 phenyl 3H 1 4-benzodiazepine-4 c
D M	7-chloro-2 amino-5 phenyl 3H 1 4-benzodiazepine-4-oxide
"Lactam"	7 chloro-1 3-dihydro-5 phenyl-2H 1 4-benzodiazepine 2

Analysis

The specific determination of the metabolites D M and lactam was done according to the method of SCHWARTZ & POSTMA (1966) The total concentration of chlor diazepoxide and D M was estimated by a spectrophotometric method measuring the absorption at 248 nm in 7 N H_2SO_4

Procedure

300 µl plasma was added to 2 ml 1 0 M potassium phosphate buffer pH 7 0–7 2 and extracted with 6 00 ml ether for 20 min, followed by centrifugation (2500 r p m.) for 5 min Lactam was removed from the ether by extraction of 5 00 ml of this phase with 3 00 ml 0 1 N NaOH for 10 min followed by the usual centrifugation In this way lactam was separated in the 0 1 N NaOH from chlordiazepoxide and D M which were still found in the ether The last mentioned compounds were removed from 4 00 ml of the ether phase by extraction with 4 00 ml 7 N H SO_4 for 10 min

Lactam analysis

The 0 1 N NaOH extract was washed with 4 ml ether and placed under a daylight lamp for 1 hour The fluorescence intensity was read at 374 nm/470 nm (uncorrected) on an Aminco Bowman spectrophotofluorometer

D M analysis

After removal of the ether by aspiration from the 7 N H SO_4 extract 1 2 ml of the extract was removed to other tubes After 1 hour the fluorescence was determined at 363 nm/470 nm (uncorrected)

Chlord azepoxide a ıalysis

The absorption in the rema ning 7 N H_2SO_4 extract was measured using a Bechman DU spectrophotometer at a wavelength of 248 nm in which both chlordiazepoxide and D M show an absorption maximum

Conformity with Beer s law was ensured for both compounds Concentrations in the plasma of treated animals were based on authentic standards of chlord azepoxide D M and lactam added to plasma and carried through the same procedure as the un knowns All determinations were done in duplicate The variation from the mean was found to be reproducible within 2 3 % The sensitivity using 300 µl plasma was 0 3–0 5 µg/ml plasma for D M and lactam and 1 2 µg/ml plasma for chlordiazep oxide Recoveries for the components ranged from 85 to 100 per cent Calculations were done according to the method of SCHWARTZ & POSTMA (1966)

Treatment

A Relative potency of chlord a epoxide D M and lactam"

Various doses of D M and lactam were injected intraperitoneally into previously trained m ce The performance tests on the rotacone were taken ½ hour later Im mediately after the test the mice were decapitated and blood samples were collected in heparinized tubes w th blood from 2 mice in each sample Plasma analys s were made as described under analysis SKF 525A 50 mg/kg was injected intraperitoneally into trained mice ½ hour before the inject on of 35 mg/kg chlord azepoxide HCl Per formance tests were carried out 10 30 or 60 min after the injection of chlordiazepox de HCl Immediately after the test the mice were decapitated and blood samples were collected in heparinized tubes with blood obtained from 2 mice in each Analysis were done according to the above procedure Chlordiazepoxide D M and SKF 525A were

Table 1

Treatment sequence

Group	1st day	24 hrs later
"Acute 50"	0 9 % NaCl 0 01 ml/g	50 mg CDZ HCl/kg 0 01 ml/g
"Repeated 50+50"	50 mg CDZ HCl/kg 0 01 ml/g	50 mg CDZ HCl/kg 0 01 ml/g
"Acute 165	0 9 % NaCl 0 01 ml/g	165 mg CDZ HCl/kg 0 01 ml/g
"Repeated 165+165"	165 mg CDZ HCl/kg 0 01 ml/g	165 mg CDZ HCl/kg 0 01 ml/g

CDZ HCl = Chlordiazepoxide,HCl

dissolved in 0 9 % saline as the hydrochloride salts "Lactam" was dissolved as the sodium salt in 0.9 % saline

B Tolerance studies

Trained mice were randomly divided into 4 groups and treated as indicated in table 1 After the first day of treatment the mice were given food and water *ad libitum* 24 hours later the normal performances of the mice on the rotacone were determined and different doses of chlordiazepoxide HCl were given intraperitoneally as indicated in the table The test and blood samples were taken as described in the SKF 525A study The groups are named as indicated in the left column In all studies the mice were kept at 31° from the injection to the test

Statistical analysis The Mann Whitney test was used for comparison of the means

Results

A Relative potency of chlordiazepoxide, D-M and "lactam" on the rotacone

From fig 2 it can be seen that the relative potencies of D M and "lactam" are almost equal

As the metabolism of chlordiazepoxide in mice is very rapid it is not possible to measure the effect of chlordiazepoxide alone in the mice Half an hour after the injection of chlordiazepoxide, HCl only small amounts of the compound are found (fig 4) In order to decrease the metabolic rate pretreatment with SKF 525A, a known inhibitor of the enzymatic degradation of many compounds, was used SKF 525A 50 mg/kg was given ½ hour before the injection of chlordiazepoxide, HCl 35 mg/kg Fig 3 shows

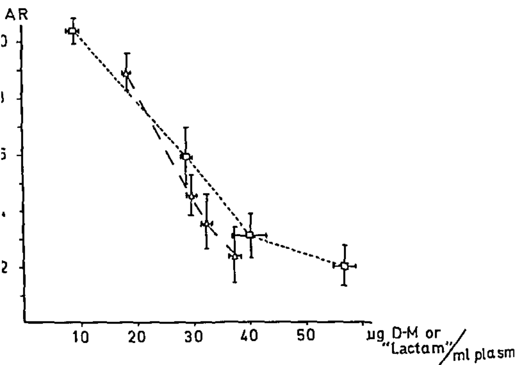


Fig 2 The performance of trained mice on the rotacone as compared with the plasma levels of D M and lactam' D M was given intraperitoneally (0 01 ml/g) in the following doses i e 30 50 60 and 80 mg/kg 'Lactam was given intraperitoneally (0 01 ml/g) in doses of 15, 30 50 and 75 mg/kg AR Each point is the mean of 14 mice ± S E M (vertival lines) Plasma levels Each point is the mean of 7 estimation ± S E M (horizontal lines) on blood from two mice after a particular treatment Test and blood samples 30 min after the injection

D M △— — — —△
Lactam □- - - - - - - -□

the plasma levels and the performances 10, 30 and 60 min after the injection of chlordiazepoxide, HCl Comparison of this figure with fig 4 shows that more chlordiazepoxide and less D M is present in the plasma of mice pretreated with SKF 525A than in the plasma of mice receiving only chlordiazepoxide, HCl at all times tested After 30 min the AR in the SKF 525A pretreated animals is low, 0,18 The plasma levels of chlordiazepoxide and D-M are 12 µg/ml plasma and 7 µg/ml plasma, respectively The "lactam" level is not measurable As seen in fig 2 37 µg D-M/ml plasma had an AR of 0 24, which indicates that chlordiazepoxide must be the most potent of the three compounds No effect was found after 50 mg/kg SKF 525A as compared to saline treated animals After 75 mg/kg SKF 525A a significant decrease

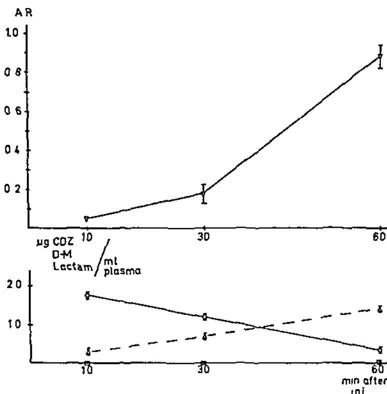


Fig 3 ½ hour after intraperitoneal injection (0 01 ml/g) of SKF 525A 50 mg/kg 35 mg/kg chlordiazepoxide HCl is given intraperitoneally (0 01 ml/g) to trained mice Plasma levels and performance on the rotacone are given as a function of time after chlordiazepoxide, HCl injection AR Each point is the mean of 16 mice ± S E M (vertical lines) Plasma levels Each point is the mean of 8 estimations on blood from two mice ± S E M (vertical lines)

Chlordiazepoxide O————O
D M △— — —△
"Lactam" □--------□

in performance was seen A synergism between SKF 525A and chlordiazepoxide is not likely with the dose of SKF 525A used, but this cannot be excluded

B Tolerance studies

Fig 4 shows the results from the "Acute 50" and the "Repeated 50+50" groups The repeatedly treated animals have a significantly higher performance ($P < 0\ 01$) 10 and 30 min after injection At the same time significant differences were found between the chlordiazepoxide and

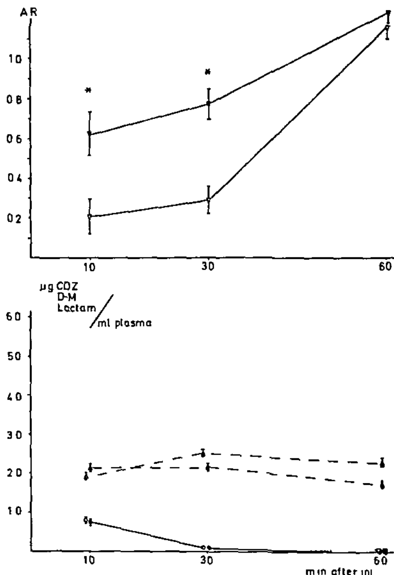


Fig 4 'Acute 50' and 'Repeated 50 + 50 Plasma levels and performance on the *rotacone as a function of time after chlordiazepoxide, HCl injection on the test day* AR Each point is the mean of 16 mice ± S E M (vertical lines) Plasma levels Each point is the mean of 8 estimations on blood from two mice ± S E M (vertical lines)

* $P < 0.01$ Mann Whitney s test
CDZ = chlordiazepoxide

	AR	CDZ	D M	"Lactam"
"Acute 50"	—▽—	—○—	—△—	---□---
"Repeated 50 + 50"	—▼—	—●—	—▲—	---■---

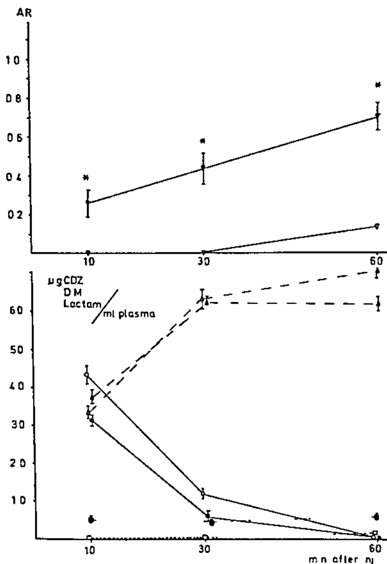


Fig 5 "Acute 165" and Repeated 165+165" Plasma levels and performance on the rotacone as a function of time after chlord azepoxide HCl injection on the test day AR Each point is the mean of 16 mice ± S E M (vertical lines) Plasma levels Each point is the mean of 8 estimations on blood from two mice ± S E M (vertical lines)

* P < 0 01 Mann Whitney s test
CDZ = chlordiazepoxide

	AR	CDZ	D M	Lactam"
"Acute 165"	—▽—	—○—	—△—	- - -□- - -
"Repeated 165+165	—▼—	—●—	—▲—	- - -■- - -

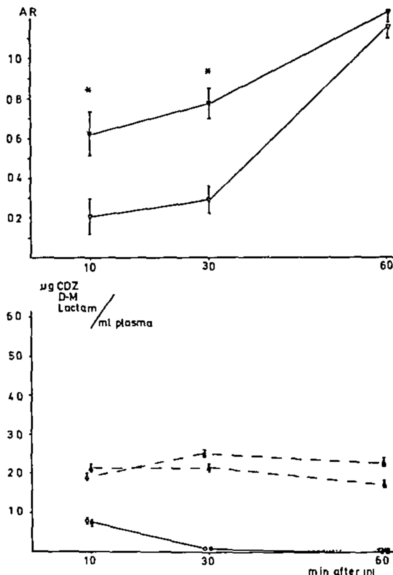


Fig 4 "Acute 50 and Repeated 50 + 50" Plasma levels and performance on the rotacone as a function of time after chlordiazepoxide HCl injection on the test day AR Each point is the mean of 16 mice + S E M (vertical lines) Plasma levels Each point is the mean of 8 estimations on blood from two mice ± S E M (vertical lines)

* P < 0 01 Mann Whitney s test
CDZ = chlordiazepoxide

	AR	CDZ	D M	"Lactam"
"Acute 50"	—▽—	—○—	—△—	---□---
"Repeated 50 + 50"	—▼—	—●—	—▲—	---■---

tam" levels The plasma level of D-M, however, after 10 min was found to be significantly higher ($P < 0.01$) in the "Repeated 50+50" group, while it was lower after 30 and 60 min ($P < 0.01$) indicating a more rapid disappearance of the compound from the plasma

Tolerance was also developed after repeated treatment with 165 mg/kg chlordiazepoxide, HCl as is seen in fig. 5, where the AR for the "Repeated 165+165" group was significantly higher than for the acutely treated animals 10, 30 and 60 min respectively after injection The plasma levels of chlordiazepoxide from the "Repeated 165+165" group compared with the "Acute 165" group are significantly lower 10 ($P < 0.01$) and 30 min ($P < 0.01$) after injection, and after 60 min the D M level is also lower ($P < 0.01$) "Lactam" levels were found to be higher in the repeated group at all testing times The overall picture shows an increased disappearance of chlordiazepoxide and D M in the repeated groups, while the "lactam" level is a little higher

These changes in the metabolism of chlordiazepoxide cannot explain the tolerance seen in this study From fig 6 which shows the results for the "Acute 50" and the "Repeated 165+165" groups it can be seen that the AR is of the same magnitude in the 2 groups 10 and 30 min after the injection At the same times the levels of chlordiazepoxide, D-M and "lactam" are significantly higher in the "Repeated 165+165" group than in the "Acute 50" group This suggests that the tolerance developed cannot solely be due to increased plasma disappearance of chlordiazepoxide and its metabolites in mice pretreated with chlordiazepoxide, HCl, not even if the relative potencies of chlordiazepoxide, D-M and "lactam" are taken into consideration

Discussion

Various mechanisms may be involved in the development of tolerance They can be divided into two classes (Kalant *et al* 1971) The first one, which is dispositional tolerance, involves factors which decrease the concentration of the drug at the site of action, such as changes in absorption, distribution, excretion and metabolism The second one, namely functional tolerance, is related to a decrease in the sensitivity of the target tissue to the drug

The present work shows that a single pretreatment with chlordiazepoxide, HCl to mice is sufficient to increase the disappearance of chlordiazepoxide and its major metabolites from the plasma as compared with acutely treated animals and in this way participates in the underlying mechanism for the tolerance developed This is, however, not sufficient to account for the tolerance developed Fig 6 shows that mice receiving a single dose of

50 mg/kg chlordiazepoxide HCl have the same performance as mice treated repeatedly with 165 mg/kg chlordiazepoxide, HCl Nevertheless the plasma levels of chlordiazepoxide and its metabolites are substantially higher in the repeatedly treated group than in the acutely treated animals

The possibility of an altered distribution from blood to brain in tolerant and non tolerant animals cannot be excluded As mentioned in the review by KALANT *et al* (1971) some disagreements can be found in the literature concerning this problem with regard to barbiturates and minor tranquilizers

The data about chlordiazepoxide of HOOGLAND *et al* (1966) show that the brain plasma ratio for chlordiazepoxide 2-^{14}C is lower in rats made tolerant to the compound than in non tolerant rats

It would be interesting to see if the same phenomena could be observed in mice and if this could explain the tolerance seen after repeated treatment with chlordiazepoxide

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Temperature-Dependent Sensitivity of Adrenoreceptors in the Toad's Heart

By

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Abstract A decrease of the experimental temperature from 25° to 12° resulted in a 10 2 × increase in the sensitivity of the toad s heart to the positive chronotropic effect of adrenaline, measured as ED50 ratios, and in a 4 9 × increase of the sensitivity to the α adrenergic drug phenylephrine In contrast, the sensitivity to the β adrenergic drug isoprenaline did not change significantly with temperature alteration Acclimatization of the animals to 25° increased the sensitivity of the heart to adrenaline by 2 2 × compared wih the 10° maintained toads The blocking capacity of the β adrenergic blocking agent, propranolol, was decreased with cold The most pronounced decrease was seen in the blocking capacity to the effect of isoprenaline On the other hand the blocking capacity of the α adrenergic blocking agent phenoxybenzamine was decreased with warmth In this case the most marked decrease was observable during antagonism between phenoxybenzamine and phenylephrine

Key words Temperature – sensitivity – adrenoreceptors – heart

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Some recent studies in mammals (GARB & PENNA 1956, TRENDELENBURG 1968, OPPERMANN *et al* 1969, 1971, 1972, REINHARDT *et al* 1972, SCHUMAN *et al* 1972) and in amphibians (KUNOS & SZENTIVÁNYI 1968, BUCKLEY & JORDAN 1970) have shown that the sensitivity of isolated organs to sympathomimetic amines is increased as the experimental temperature is decreased Furthermore, it is shown that in the isolated frog heart the blocking capacity of α adrenergic blocking agents to the effect of adrenaline is increased at low temperatures, whilst the blocking capacity of β adrenergic blocking agents is decreased (KUNOS & SZENTIVANYI 1968, BUCKLEY & JORDAN 1970) However, on isolated guinea pig atria, the affinity of β blockers was not influenced by temperature changes (REINHARDT *et al* 1972)

In this study the sensitivity of the toad's heart to the positive chronotropic effect of sympathomimetics and the capacity of adrenergic blocking agents to antagonize these effects was tested at two temperatures using an *in vivo* technique In addition, the importance of temperature acclimatization in modulating the sensitivity of the heart to adrenaline was studied

Materials and Methods

Animals

Altogether 101 male summer toads (Bufo bufo), weighing from 30 to 45 g were used The animals were maintained at 10° for at least two weeks before use Some animals were also acclimatized to 25° for two weeks before assay

Experimental procedure

The toads were pithed The chest was opened leaving the pericardium undamaged A polyethylene tubing combined with an injection syringe was introduced into the abdominal vein for the injection of drugs, and the animals were allowed to stabilize for 30–50 min before addition of the drug at the experimental temperatures of 25° and 12° The abdominal vein was cut distal to the tubing immediately before the agonist injection in order to allow any excess fluid to flow out Cumulative dose response relationships were determined by the injection of agonist at intervals of 2 min and by increasing the concentration of agonist in the injection fluid in steps of 0 5 log units until no further increase in positive chronotropic response to that agonist had developed The heart rate was counted during the 30 to 90 second period post injection, at which time the maximum response had already developed When the blocking capacity of an adrenergic antagonist was studied, the antagonist was dissolved in the same frog Ringer as the agonist In order to achieve a sufficient concentration of antagonist at the receptor sites even before the agonist injection Ringer containing the antagonist in a concentration indicated in table 1, was injected 30 and 5 min before the agonist The injected volume was 0 04 ml/10 g of body weight The dose of agonist required to produce 50 % of the maximum positive chronotropic response (ED50) was determined from the log dose response curve obtained A single dose response relationship was determined on each heart preparation Significance of differences was estimated by Student s t test

Drugs

The following drugs were used l adrenaline as base (Merck A G) isoprenaline hydrochloride (isuprel®, Winthrop) phenylephrine chloride (metaoxedrinum NFN) (neosynephrine®, Winthrop), propranolol chloride (proprasylytum NFN) (inderal®, I C I), phenoxybenzamine chloride (bensylytum NFN) (dibenyline®, Smith, Kline and French Laboratories)

Results

The basal heart rates were 24 2 ± 1 24 beats/min at 25° and 13 4 ± 0 33 beats/min at 12° The results in fig 1 and in table 1 show that a decrease in the experimental temperature from 25° to 12° resulted in a 10 2 times

Table 1

Effect of experimental temperature and temperature acclimatization on the sensitivity of the toad's heart to the chronotropic effect of adrenergic agonists and antagonists. Agonists were injected intravenously with or without antagonists. The concentration of antagonists in the injection fluid is indicated. Given are mean values ± S.E.M., number of experiments in brackets

Treatment	25°		12°	
	ED50 (μg/kg)	Maximum response (beats/min.)	ED50 (μg/kg)	Maximum response (beats/min.)
10° acclimatized toads				
Adrenaline	2.94 ± 0.24	24.5 ± 3.2 (7)	0.29 ± 0.06	12.3 ± 0.6 (5)
+ Propranolol 3×10^{-7} M	9.84 ± 2.88*	26.7 ± 1.6 (8)	1.35 ± 0.44*	11.3 ± 0.7 (5)
+ Phenoxybenzamine 5×10^{-6} M	12.20 ± 2.49**	32.8 ± 2.0 (4)	2.00 ± 0.66**	12.4 ± 0.8 (4)
Isoprenaline	0.30 ± 0.03	25.3 ± 1.3 (4)	0.22 ± 0.03	13.2 ± 1.1 (5)
+ Propranolol 3×10^{-7} M	3.21 ± 0.38***	33.3 ± 2.0 (5)	0.57 ± 0.14*	11.7 ± 0.9 (5)
+ Phenoxybenzamine 5×10^{-6} M	0.70 ± 0.17	29.6 ± 1.9 (4)	0.19 ± 0.03	12.0 ± 0.8 (5)
Phenylephrine	48.80 ± 14.7	20.2 ± 4.0 (5)	9.96 ± 2.68	10.3 ± 0.6 (6)
+ Propranolol 3×10^{-7} M	191.20 ± 33.6**	19.3 ± 2.0 (4)	10.76 ± 3.04	6.9 ± 1.5 (5)
+ Phenoxybenzamine 5×10^{-6} M	62.80 ± 9.60	20.0 ± 1.7 (6)	131.20 ± 31.6**	7.0 ± 2.0 (5)
25°-acclimatized toads				
Adrenaline	1.34 ± 0.16	29.5 ± 3.8 (6)		

Significant difference from the ED50 values of agonist without antagonist, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

decrease of the ED50-values for the positive chronotropic response of the toad's heart to adrenaline ($P < 0.001$) and in a 4.9 times decrease to phenylephrine ($P < 0.02$). Thus, the sensitivity of the toad's heart to these drugs was increased with cold. In contrast, the sensitivity to isoprenaline did not change significantly with temperature alteration. The maximum responses to these drugs did not differ significantly from each other either when measured at 25° or at 12°. The results also show that acclimatization of the 10°-maintained toads to 25° shifted the dose-response curves for adrenaline to the left by 0.22 log units ($P < 0.001$).

Addition of adrenergic blocking agents to the injection fluid shifted the dose-response curves for the agonists to the right in a parallel manner at both temperatures used, without reducing the maxima. Propranolol in 3×10^{-7} M concentration in the injection fluid increased the ED50 values for adrenaline by 3.5 times when measured at 25° and by 4.7 times when measured at 12°. These values do not differ significantly from each other. However, the blocking capacity of 3×10^{-7} M propranolol to the effect of isoprenaline

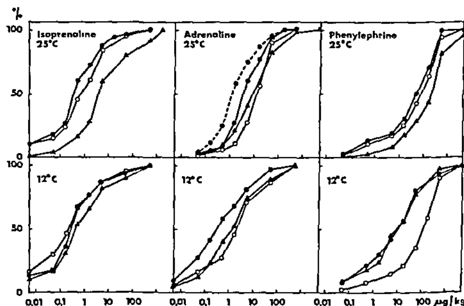


Fig 1 The log dose response curves for the positive chronotropic responses of the toad's heart to isoprenaline, adrenaline and phenylephrine when measured at 25° and at 12° without antagonists (dots) and with 3×10^{-7} M propranolol (triangles) or 5×10^{-6} M phenoxybenzamine (open circles) in the injection fluid. Continuous lines indicate +10° acclimatized and broken lines +25° acclimatized toads. The responses are illustrated in percentage of maximum (= 100 %). The exact values of the maximal responses and the numbers of experiments are given in table 1

was decreased from 10 7 times at 25° to 2 6 times at 12° and that to the effect of phenylephrine from 3 9 times to 1 1 times, respectively, as measured by the ED50 ratios in the presence and absence of the blocking agent. A smaller concentration of propranolol (3×10^{-8} M) did not affect significantly the ED50 values for the amines tested The blocking capacity of 5×10^{-6} M (smaller concentrations were ineffective) phenoxybenzamine to the effect of phenylephrine was 10 2 times greater at 12° than at 25°. Furthermore, phenoxybenzamine caused a 6 9 times blockade to the effect of adrenaline at 12° but only a 4 2 times blockade at 25° In contrast phenoxybenzamine did not block significantly the response of the toad's heart to isoprenaline at any of the temperatures used

Discussion

The *in vivo* technique used proved to be a very simple and rapid method for testing the drug effects on the amphibian heart, since the drugs injected into the abdominal vein reached the heart rapidly and practically without dilution Cumulative dose-response curves, generally used for isolated organs only, were regarded as the most useful method for comparing the potencies of different sympathomimetic amines, since the duration of the chronotropic response caused by a single amine injection remained unaltered for at least 5 min even at the higher experimental temperature In this study the maximum responses of the toad's heart to the sympathomimetics used did not differ significantly from each other at any of the temperatures used Hence the ED50-values gave the best results for comparing the potencies of different agonists In agreement with these results the maximal developed inotropic effects induced by some β-adrenergic drugs on guinea pig atria did not differ from each other at temperatures of 25° and 33° (REINHARDT *et al* 1972) On the other hand, BUCKLEY & JORDAN (1970) observed that the extent of the effect of isoprenaline on work output (force X rate) of the isolated frog heart was decreased at low bath temperatures more than those of adrenaline or noradrenaline However, they used only a few doses (2 to 3) so that one cannot draw any conclusions Furthermore, in agreement with this study KUNOS & SZENTIVANYI (1968) showed that in the isolated frog heart, the treshold concentration to adrenaline was decreased at low bath temperatures On the other hand, there are data which show that the temperature-dependent sensitivity of isolated auricles might depend on the animals species used (OPPERMANN *et al* 1969)

OPPERMANN *et al* (1971, 1972) concluded that the temperature-dependent sensitivity of isolated guinea-pig atria may be due to decreased catechol-O-methyl transferase activity at the lower bath temperature but not to a

decreased uptake of amines It is, however, interesting to note that the affinity of the amines used for the uptake mechanism in rat heart is adrenaline > phenylephrine > isoprenaline (IVERSEN 1964) If a decrease in the activity of the uptake mechanism occurs at the lower temperature one would expect to see changes in sensitivity in the same relative order, which in fact is the case in this study Some reports based on experiments in mammals (REINHARDT *et al* 1972, SCHUMAN *et al* 1972) and in frogs (KUNOS & SZENTIVANYI 1968 BUCKLEY & JORDAN 1970) support the assumption that the metabolic rate of an organ may be the factor which determines the sensitivity of the adrenoreceptors and hence the selectivity of the sympathomimetic drugs This is also supported by the observation that acclimatization to warmth changed the sensitivity of the toad s heart to adrenaline Investigations are in progress to elucidate in more details the role of temperature acclimatization in modulating the sensitivity of adrenoreceptors

In the isolated frog heart the blocking capacity of α adrenergic blocking agents to the effect of adrenaline was found to increase at low temperatures the opposite being the case for the blocking capacity of β blockers (KUNOS & SZENTIVANYI 1968, BUCKLEY & JORDAN 1970) On the other hand, in the isolated guinea pig atria the affinity of β blockers to the effect of β adrenergic drugs was not influenced by temperature changes (REINHARDT *et al* 1972) In this study the blocking capacity of propranolol (β blocker) was decreased with cold, the most pronounced decrease was seen in the blocking capacity of propranolol to the effect of isoprenaline (β stimulant) On the contrary the observed increase in the blocking capacity of phenoxybenzamine (α blocker) in the cold was most marked in the phenoxybenzamine to phenylephrine (α stimulant) antagonism It can be concluded that the affinity of α blockers on the adrenoreceptors of the toad s heart is increased at low temperatures whilst the affinity of the β blockers is increased at high temperatures

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Biliary Excretion and Intestinal Reabsorption of Mercury in the Rat after Injection of Methyl Mercuric Cloride

By

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(Received March 9, 1973, Accepted April 4, 1973)

Abstract: The relative amount of methyl mercuric compounds in rat bile is not dose dependent for doses from 1 mg Hg/kg rat weight to less than 1 μg Hg/kg given as methyl mercuric chloride. Binding of mercury in the bile and reabsorption from the intestinal tract are similar for doses several orders of magnitude apart. The reabsorption of mercury is not part of an enterohepatic circulation as mercury which is reabsorbed is not predominantly reexcreted. The distribution of sulfhydryl groups between the liver and the bile does not explain the distribution of mercury. The excretion of mercury does not increase when the volume of bile increases. The mercury concentration in the bile reflects the liver concentration and not the plasma concentration. There have been no indications of an active transport mechanism for methyl mercuric compounds from the liver cell to the bile demonstrated.

Key words: Methyl mercuric chloride – biliary excretion – entero-hepatic circulation.

Mercury is excreted in the bile in a number of animal species after exposure to methyl mercuric salts (Norseth & Clarkson 1971, Norseth 1971a, Vostal 1972). The biliary content of mercury both in the rat and in the mouse after such exposure is higher than the faecal excretion thus indicating reabsorption of biliary mercury from the gastro-intestinal tract (Norseth & Clarkson 1971, Norseth 1973). Mercury in bile after exposure to methyl mercuric salts is bound to a number of different compounds, including at least one protein compound and one compound of the molecular size of a small peptide or an amino acid (Norseth & Clarkson 1971, Norseth 1971b). The rate of reabsorption of mercury is different for the different compounds, the small molecular compound being reabsorbed to the highest extent. Based on this observation, peroral treatment of mice after exposure to methyl mercuric chloride with a resin which could not be absorbed was suggested. Several resins substituated with sulfhydryl

groups were tested, and a polyvinyl resin was found to be effective (CLARKSON *et al* 1973)

The studies on the intestinal transport of methyl mercury in the rat have so far been done in a model system by administering a standard dose of 1 mg Hg/kg rat weight as methyl mercuric chloride For practical toxicological purposes *this must be assumed to be an unrealistic high dose Furthermore*, to what extent the reabsorption really demonstrates an enterohepatic circulation involving reabsorption and subsequent reexcretion in the bile is not known

In this paper therefore the relative biliary excretion, reexcretion and binding of mercury in bile after the intravenous injection of different doses of methyl mercury chloride has been tested

Materials and methods

A single intravenous injection of ^{203}Hg labelled methyl mercuric chloride was given to female rats (mean weight 200 g range 180 g – 230 g) of our own breed (Institute of Occupational Health Wistar) in doses ranging from 5 mg Hg/kg rat weight (1000 µg/rat) to 0 001 mg Hg/kg rat weight (0 2 µg/rat) The rats were kept in barbiturate narcosis during a two hours collection period A tracheal tube was inserted to keep free airways and the body temperature was kept constant The bile was collected in pre weighed vials and the amount of bile was determined by weighing The mercury concentration was determined by counting each vial and compaired with the standard samples of the injection solution with known specific activity

A system including two rats with canulated bile ducts was used for testing the extent of reexcretion of mercury Bile from one rat was introduced into the duodenum of the other rat over 5 hours and bile from this rat was collected as described previously at intervals of 15 min At the end of the experiment both rats were counted in a whole body counter the intestinal tract from the rat receiving bile was removed and counted and the biliary excretion of mercury from this rat determined as previously described

The effect of bile flow on mercury excretion was tested after inducing a high flow of bile by sodium dehydrocholate treatment Sodium dehydrocholate was injected subcutaneously after a control excretion period of 2 hours and the excretion tested at 15 min intervals over the first hour and at 60 min intervals for an additional 3 hours period

Rats were given 1 mg Hg/kg as methyl mercuric chloride and killed at the time intervals of 1 hour 2 hours 4 hours and 24 hours to test the ratio of biliary concentration of mercury to that of the liver plasma or blood The concentration of mercury in samples of blood plasma and liver homogenates was determined as described for bile

The binding of mercury in bile was determined by column chromatography on sephadex G 75 in 0 1 M Tris buffer pH 8 0 and with sodium azide and 0.5 M NaCl added Fractions of 5 ml were collected and the exclusion volume determined using blue dextran (AB Pharmacia)

Methyl mercuric cysteine was used for indicating the elution of the small molecular compound even if final identification of this compound had not been done Chromato-

Table 1

Mercury excreted in bile with different doses

Dose µg/rat	No of rats	Excretion (mean ± S D) µg/2 hours	% of dose
1000	2	20 1431	2 0
200	45	3 9240 ± 1.2977	1 9 ± 0 6
20	4	0 2700 ± 0 0837	1 1 ± 0 8
2	4	0 0583 ± 0 0134	3 2 ± 1 0
0 2	5	0 0050	2 5 ± 0 6

graphy was performed shortly after the bile was collected but some samples kept frozen or cooled were run the next day for control purposes Mercury in the fractions was determined by direct counting of the fractions with standards and adjusted for decreased counting efficiency because of large counting volumes

Sulfhydryl groups in the liver and bile were determined by the method of SEDLAK & LINDSAY (1968) The liver was homogenized in ice cold 0 02 M EDTA For non protein sulfhydryl groups proteins were presipitated with 10 % of 50 % TCA Bile was collected directly into the reaction mixture or into ice-cold 0 02 M EDTA

Results

The relative amount of the injected dose of mercury excreted in the bile during the first two hours varied from 1 1 % to 3 2 % of the dose (table 1) The variation did not, however, seem to be dependent on the injected dose The highest and the lowest dose resulted in similar relative excretion The collection period of two hours was used because these rats were also used for other purposes Longer time intervals were tested for some doses The time course of the excretion did not vary with the dose

Table 2

Enterohepatic circulation of mercury

No of rat pairs	Dose (µg/rat)		Biliary excretion (mean ± S D) % of dose	
	Rat 1[a]	Rat 2[b]	Rat 1	Rat 2
3	200	14	7 1 ± 0 4	4 8 ± 2.5
3	0 2	0 014	7 2 ± 1 2	3 9 ± 1 8

a) The dose was given by intravenous injection

b) The dose was given by implantation of the canulated bile duct from one injected rat into the duodenum of one of this group The value corresponds to biliary excretion from the injected group

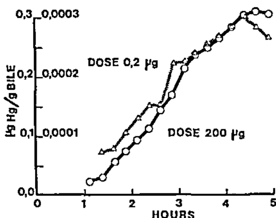


Fig 1 Biliary excretion of mercury in rats given mercuric chloride by direct infusion into duodenum of the bile from rats injected with methyl mercuric chloride Circles represent an injected dose of 200 µg Hg/rat, and the corresponding excretion values are given from 0 0 to 0 3 µg Hg/g bile Triangles represent an injected dose of 0 2 µg Hg/rat, and the excretion values are given from 0 0 to 0 0003 µg Hg/g bile The rats were injected at time 0, and each point represents the mean of 2 experiments

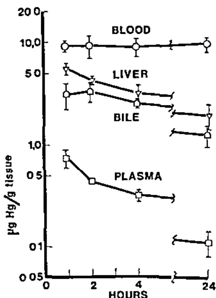


Fig 2 Mercury concentration in rat organs at different time intervals after intra venous injection of 200 µg Hg/rat as methyl mercuric chloride Each point represents mean ± S D for 3 to 5 rats

When bile from rats given 0 2 µg of mercury was directly excreted into the duodenum of another rat, 7 2 % of the original dose was excreted during 5 hours Biliary excretion of mercury from the second rat was 3 9 % of the introduced dose (table 2) When the dose given was 200 µg 7 1 % was excreted by the treated rat while 4 8 % was excreted by the rat receiving bile

There was a lag period of about 60 min between treatment of the first rat before the mercury could be detected in the bile of the second rat This lag period as well as the time course of the excretion was the same for both the dose levels tested (fig 1)

The biliary concentration of mercury was about 3 µg/g both after 1 and 2 hours, then decreased to about 2 5 µg/g after 4 hours and slightly more than 1 µg/g after 24 hours (fig 2) The concentration of mercury in the

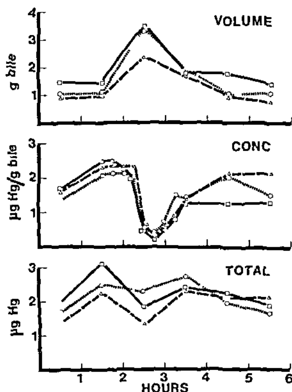


Fig 3 Biliary excretion of mercury in rats given a single intravenous injection of methyl mercuric chloride at time 0 followed by 100 mg sodium dehydrocholate after 2 hours Concentration of mercury in bile is given for intervals of 15 min over the hours before and after the dehydrocholate injection Average values for the same time periods are given for bile volume and total mercury excretion Each curve represents one rat

liver was after 1 hour between 5 and 6 μg/g, then fell to about 4 5 μg/g, 3 μg/g and 1 5 μg/g after 2, 4 and 24 hours, respectively There were no differences between the liver and bile concentrations after 2, 4 and 24 hours (t test, $P < 0\ 05$)

The blood concentration of mercury was slightly less than 10 μg/ml during the testing period (fig 2) Plasma concentrations fell from about 7 μg/ml to 0 1 μg/ml during the same period The red cell to plasma ratio increased from 24 after 1 hour to 138 after 24 hours

The biliary concentration of mercury decreased after treatment with de hydrocholate The bile volume increased as expected, and the total mercury excretion was unaltered (fig 3)

Mercury in bile is bound to some small molecular compound independently of the dose as no difference was found for the doses of 200 μg and 0 2 μg (fig 4) When the bile was stored for 24 hours there was a shift in the binding and all the mercury was found in a fraction corresponding to some high molecular weight compound when 0 2 μg was given A shift in binding was also found with the high dose, but only a small part of the mercury shifted with this dose

Total sulfhydryl groups in bile were 2 33 ± 0 24 μmol/g (n = 16) The amount of protein sulfhydryl in bile was too low to be determined by the method used as no differences were found between the non protein and the total amount Total sulfhydryl groups in the liver were found to be

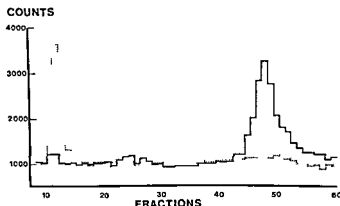


Fig 4 Column chromatography on sephadex G 75 in 0 1 M tris buffer pH 8 0 of bile collected during the first 2 hours after an intravenous injection of 0 2 μg Hg as methyl mercuric chloride Blue dextran and methyl mercuric cysteine were eluted corresponding to fractions 10–13 and 46–50 respectively The drawn curve represents bile applied to the column shortly after the collection period was ended the dotted line represents an aliquot of the same sample after being stored frozen for about 24 hours

21 86 ± 2 47 μmol/g (n=8) and the non-protein part was 4 66 ± 0 92 μmol/g (n = 5) Sulfhydryl groups in bile and non protein sulfhydryl groups in the liver are significantly different (t-test, $P < 0\ 001$)

Discussion

The biliary excretion and reabsorption patterns demonstrated previously for relative high doses of methyl mercury chloride in the rat are the same for doses several orders of magnitude apart (NORSETH & CLARKSON 1971) This indicates that treatment with resins which are not absorbed will also act with low doses of mercury (CLARKSON *et al* 1973) The effect of the treatment will, however, depend on the relative amount of mercury excreted in the bile of different animal species Differences in mercury excretion between species have been demonstrated (VOSTAL 1972) Mercury has been demonstrated in human bile, but both exposure conditions and the mercury compound used are unknown (NORSETH, unpublished results)

Mercury in bile after exposure of rats to methyl mercuric chloride is almost exclusively present as methyl mercuric salts, a small amount being inorganic mercury (NORSETH & CLARKSON 1971) The mechanism of the methyl mercury excretory process is unknown No indication of a saturable process has been demonstrated with doses up to 5 mg Hg/kg rat weight or 1000 μg/rat, corresponding to about 10 μg Hg/g of bile Both the results from five dose levels tested for the two hour period, and the excretion and re excretion experiment which includes four dose levels for the excretion, support this conclusion Even the concentration 10 μg Hg/g, however, is lower by more than a factor of ten on a molar basis than the concentration of the sulfhydryl groups in bile

The intestinal transport of mercury does not seem to be part of a real enterohepatic circulation if the term includes re-excretion of the amount absorbed The relative amount of mercury re-excreted as compared with the biliary excretion after intravenous injection, cannot be accurately estimated because of the different exposure pattern A lower amount of the body burden, rather than a higher amount seems to be the correct answer This assumption is correct even with a degree of reabsorption of about 65 %, a value recorded for some of the rats

Whether the binding pattern in bile reflects the excretory mechanisms or unspecific binding is not known The same problems related to biliary manganese excretion has been discussed by TICHY & CIKRT (1972), but the binding of the methyl mercuric ion seems to be more specific in the bile than the binding of manganese Even with the high stability constant of mercury for sulfhydryl groups, there is a re-distribution in the bile when samples are stored This re-distribution is obvious only with low concentra

tions of mercury in the bile, and it probably reflects a small amount of protein sulfhydryl groups, in the range of mμmol/g bile

The biliary excretion of methyl mercuric salts cannot be explained by transfer of the metal to a compartment with a higher concentration of sulfhydryl groups the bile being this compartment The transport may be related to the transport of some small molecular compound with a sulfhydryl group A compound corresponding to the methyl mercuric compound demonstrated in bile could not be found by column chromatography in liver cell homogenates or in the nuclear, the mitochondrial, the lysomal, the microsomal or the supernatant from the liver cell (NORSETH, unpublished results) Small molecules with sulfhydryl groups present in the liver indicate that such a compound may exist in small amounts probably with a rapid turnover

The similar concentration of mercury in the liver and in the bile, and the similar relative excretion over the doses tested do not indicate an active process for the transport of mercury to the bile For the first two hours there is a rapid rise in biliary mercury concentration but when the concentration reaches the same level as for the liver, the concentration falls at the same rate as for this organ

The low plasma values of mercury as demonstrated previously was found in this investigation but the blood plasma ratio was considerably higher shortly after the intravenous injection than after 24 hours (NORSETH & CLARKSON 1971) This did not influence the biliary excretion of mercury, but may be of importance in explaining the liver uptake There is a high concentration gradient between plasma and liver, but the mechanisms of liver uptake is not known

Passive transfer and excretion of mercury dependent on bile flow were not found The concentration of sulfhydryl groups in bile decreased corresponding to the increased volume of bile Sulfhydryl groups and mercury in bile were thus altered in a similar way by dehydrocholate treatment

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From the Toxicology Laboratories AB Astra, Sodertälje, Sweden

Uptake of Piribenzil by Rat Liver Slices

By

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Abstract The uptake of ^3H piribenzil (2 hydroxymethyl) 1,1 dimethyl piperidinium (^3H) benzilate an anticholinergic agent into rat liver slices was found to be concentration and time dependent At a medium concentration of 10^{-5} M the slice to medium (S/M) ratio was about 4 after 60 minutes incubation The uptake was decreased by metabolic inhibitors (iodoacetic acid and 2 4-dinitrophenol) and by incubation in N_2-atmosphere Structurally similar compounds (cations) also decreased the uptake of piribenzil but organic anions did not influence the uptake process Chromatographic studies revealed unchanged piribenzil in the slices Binding of piribenzil to liver homogenate occurred Moreover efflux of piribenzil from liver slices was decreased by iodoacetic acid These *in vitro* experiments may reflect processes responsible for the biliary excretion of organic cations

Key words Piribenzil – transport systems – liver

The importance of biliary excretion for the elimination of many organic cationic compounds is well known (SMITH 1966, SCHANKER 1968) Processes such as passive diffusion, affinity for cellular components and energy dependent transport system(s) are very probably of importance for the transfer of a compound from the blood into the liver cells and out into the bile To elucidate the importance of such processes, incubation with liver slices are of interest Such studies have been performed with e g procaine amide ethobromide (PAEB) (SOLOMON & SCHANKER 1963) and chloroguanidetriazine (CGT) (GIGON *et al* 1969) These compounds are known to be eliminated in the bile to a large extent The studies with CGT indicated extensive binding to cellular components but the uptake was also energy dependent The uptake of PAEB was markedly energy dependent and the uptake could be blocked by various inhibitors Negligible binding to cellular components occurred

In order to learn more about the importance of the above mentioned processes for uptake into liver tissues and then possibly for the mechanisms

responsible for the biliary excretion of organic cations, the present study with ^3H piribenzil [2 (hydroxymethyl)-1,1-dimethyl piperidinium (-^3H)benzilate] an anticholinergic agent, was undertaken This substance is a monoquater nary ammonium compound and is known to be eliminated to a large extent in the bile (RYRFELDT & HANSSON 1971) The uptake of piribenzil was studied e g in the presence of various metabolic inhibitors and other organic compounds which might influence the uptake process

Material and methods

^3H piribenzil methyl sulphate (sp act 9 5 μci/mg) was obtained from the Research and Development Laboratories Astra Lakemedel AB Sodertalje Sweden The radiochemical purity of the compound was checked by means of thin layer chromatography as described below The compound proved to be radio chromatographically pure

All other chemicals used in this investigation were of analytical grade and supplied by E Merck AG and Fischer Scientific Co or as indicated in the text

Uptake studies

Female and male Sprague Dawley rats (250–300 g) were decapitated bled and the livers removed as quickly as possible and placed in beakers containing ice cold saline Slices were cut free hand (about 0 5 mm thick) and placed in ice cold Tyrode s solution (pH 7 4 containing 1 g glucose/l) The slices were then rinsed several times with ice cold Tyrode s solution and pre incubated for 15 minutes at 37° to get rid of weakly bound cell components

The uptake studies were performed in vials containing 2 00 ml Tyrode s solution and a liver slice (weight 70-130 mg) ^3H piribenzil was added in 0 20 ml saline In some experiments the following compounds were added to the incubation medium to study the r effect on the uptake of piribenzil 2 4-dinitrophenol iodoacetic acid ouabain (Sigma Chemical Co) sodium cyanide emepron bromide (AB Kabi Stockholm Sweden) methylatropine lidocaine (AB Astra Sodertalje Sweden) phenolphthalein glucuronide (Sigma Chemical Co) azidocillin ampicillin (both AB Astra Södertalje Sweden) sulphobromophthalein sodium (BSP) phenolphthalein and phenemal sodium These compounds were added in a volume of 0 20 ml saline The final incubation volume was in all experiments 2.40 ml Incubations were performed at 37° in carbogen atmosphere (93 5 % O_2 and 6 5 % CO_2) and the vials were shaken in a metabol c shaker To stop the incubation the vials were taken from the incubator and placed in ice cold water The slices were removed blotted on slightly moistened filter paper and trans ferred to test tubes containing 1 00 ml ice-cold water The slices were homogenized in a Potter Elvehjem homogenizer and the radioactivity of the homogenate as well as that of the medium was assayed

Results are expressed as slice to medium concentration rat os (S/M ratio) and are calculated as $\frac{\text{d p m /100 mg slices}}{\text{d p m /100 µl medium}}$

Efflux studies

Liver slices (weighing 93–107 mg) were prepared as described above Pre-incubation was performed by transferring a slice into 2 00 ml Tyrode's buffer and incubated at 37° for 10 minutes for temperature stabilization After that 0 20 ml saline containing piribenzil was added The concentration of piribenzil in the final medium was 10^{-6} M Incubation was performed for 60 minutes at 37° in carbogen atmosphere and under agitation

After this slices were blotted on slightly moisted filter paper washed in ice-cold Tyrode's solution (whole procedure 10 seconds) and transferred to vials containing either 1 00 ml Tyrode's solution or 1 00 ml Tyrode's solution containing iodoacetic acid (10^{-3} M) Incubation was then performed at 37° in carbogen atmosphere Samples (100 µl) were taken out from the medium at 10, 20 and 40 minutes after the start of the incubation, for radioactive assay

Radioactive assay

The radioactivity of the homogenate was assayed by transferring aliquots of 200 mg to counting vials and the samples were dissolved in 0 40 ml perchloric acid (70 %) followed by 0 20 ml hydrogen peroxide (30 %) The vials were placed in an oven at 70° for one hour After cooling 15 ml scintillation solution was added (7 0 g Butyl PBD/Ciba/, 600 ml ethylene glycol monoethyl ether and 1000 ml toluene) Water solutions were assayed by dissolving 100 µl in 15 ml scintillation solution

The counting efficiency was determined by external standard channels ratio procedure The counting was performed so as to avoid an error larger than ± 3 % (95 % confidence limit) in the total count of each sample

Chromatography

Pooled liver homogenates, representing the same incubation time, were centrifuged at 9 000 r p m for 15 minutes and aliquots (1 00 ml) of the supernatant were freeze-dried To the residue 0 40 ml ethanol was added and the samples were shaken for 15 minutes followed by centrifugation Aliquots (100 µl) of the supernatant were spotted on pre coated silica gel plates (E Merck AG) and the plates were developed with n butanol formic acid water (40 10 20) After developing and drying of the plate 11 zones of one cm were scraped off and the scrapings transferred to counting vials containing 0 5 ml 0 1 M KBr solution and the vials shaken for one hour Following this scintillation solution was added and the samples assayed The recovery of the radioactivity applied to the plate was 92 95 %

Ultrafiltration

The compound was dissolved in water and added to various dilutions of rat liver homogenate in water Five experiments were performed at each liver homogenate dilution

Ultrafiltration was performed at 37° using Visking tubings and at a positive pressure of 1 kg/cm² The volume of liver homogenate used was 5 ml and the volume of ultra filtrate collected was about 0 5 ml The method is described in detail by ROLINSON & SUTHERLAND (1965)

The radioactivity of the ultrafiltrate and liver homogenate was assayed as described before

Statistical treatment

Results are expressed as means ± standard deviation (S D) The level of significance was determined by Student's t test.

Results

Uptake of piribenzil

A rapid initial uptake of radioactivity is found which gradually diminishes with increasing incubation time (fig 1). When the concentration of piribenzil was 10^{-5} M, a higher uptake was noted than with a 10 times higher concentration. After 60 minutes incubation the S/M ratio was 4.15 ± 0.64 ($\bar{x}$ ± S.D., n = 12) with a concentration of 10^{-5} M and the S/M-ratio was 2.00 ± 0.28 (n = 4) with 10^{-4} M concentration of piribenzil.

No apparent difference in the uptake of piribenzil between liver slices obtained from female and male rats was found. The S/M-ratio at 60 min incubation time for females was 4.15 ± 0.64 (n = 12) and the corresponding value for male rats was 4.68 ± 0.43 (n = 6).

Effect of metabolic inhibitors and N_2 atmosphere on uptake

Table 1 and fig 2 show the effect of various metabolic inhibitors on the uptake of radioactivity. In all studies using metabolic inhibitors the slices were pre-incubated for 10 minutes before ^{3}H piribenzil was added. Both 2,4-dinitrophenol and iodoacetic acid cause significant inhibition of the uptake process ($P < 0.001$ with 10^{-3} M concentration of inhibitor). The high concentration of ouabain (10^{-3} M) causes a significant inhibition ($P < 0.05$) but at the lower concentration of ouabain (10^{-4} M) no significant

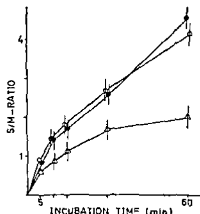


Fig 1 Influence of concentration of ^{3}H piribenzil on its uptake by rat liver slices. Comparison between slices obtained from male and female rats is also shown. The uptake is expressed as slice to medium concentration ratios (S/M value). Each point is the mean of 4–12 experiment. Brackets indicate the S.D.

○ piribenzil 1 × 10^{-5} M (♀)
△ piribenzil 1 × 10^{-4} M (♀)
● piribenzil 1 × 10^{-5} M (♂)

Table 1

Effect of various metabolic inhibitors on the uptake of ^3H piribenzil in liver slices of rats *The uptake is expressed as slice to-medium (S/M) concentration ratio after* 60 min incubation The concentration of piribenzil in the medium was 10^{-5} M Results are given as mean values from 4 experiments ± S D (control group 16 experiments)

Inhibitor	Concentrations (M)	S/M ratio	P
2,4-dinitrophenol*	10^{-4}	2 86 ± 0 11	< 0 001
	10^{-3}	2 42 ± 0 42	< 0 001
Iodoacetic acid*	10^{-3}	1 86 ± 0 34	< 0 001
Ouabain*	10^{-4}	3 74 ± 0 14	N S**
	10^{-3}	3 33 ± 0 59	< 0 02
Sodium cyanide*	10^{-3}	3 84 ± 0 72	N S
N_2-atmosphere		3 16 ± 0 16	< 0 01
Control*		4 21 ± 0 59	

* Incubations performed in carbogen atmosphere
** N S Not significant (95 % confidence limit)

inhibition was noted *Sodium cyanide does not seem to influence the uptake* process in the actual concentration (10^{-3} M)

Incubation in N_2-atmosphere does not seem to influence the uptake process at the shorter incubation times (5–15 min) However, the S/M-values obtained after 30 and 60 minutes incubation indicate a significantly lower uptake of piribenzil

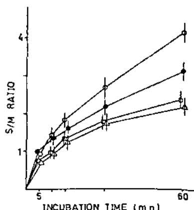


Fig 2 Effect of metabolic inhibitors and the absence of oxygen on the uptake of piribenzil by liver slices obtained from female rats Each point is the mean of 4–12 experiments Brackets indicate the S D

○ piribenzil 1 × 10^{-5} M
□ piribenzil 1 × 10^{-5} M + dinitrophenol 1 × 10^{-3} M
△ piribenzil 1 × 10^{-5} M + iodoacetic acid 1 × 10^{-3} M
● piribenzil 1 × 10^{-5} M in N_2-atmosphere

Table 2.

The effect of some organic compounds on the uptake of ^{3}H piribenzil in rat liver slices. Incubations were performed in carbogen atmosphere at 37°. The uptake is expressed as slices-to-medium (S/M) concentration ratio after 60 min incubation. The concentration of piribenzil in the medium was 10^{-5} M. Results are given as mean values from 4–8 experiments ± S.D. (control group, 16 experiments)

Compound	Concentration (M)	S/M ratio	P
Emepronbromide	10^{-4}	1.32 ± 0.09	< 0.001
	10^{-3}	1.09 ± 0.14	< 0.001
Methylatropine	10^{-4}	2.21 ± 0.30	< 0.001
Lidocaine	10^{-4}	2.88 ± 0.20	< 0.001
Phenolphthalein	10^{-4}	4.23 ± 0.71	N.S.*
Phenolphthalein glucuronide	10^{-4}	4.62 ± 0.51	N.S.
BSP	10^{-4}	4.88 ± 0.71	N.S.
Azidocillin	10^{-4}	4.03 ± 0.53	N.S.
Ampicillin	10^{-4}	3.97 ± 0.37	N.S.
Phenemal	10^{-4}	3.76 ± 0.38	N.S.
Control		4.21 ± 0.59	

* N.S. Not significant (95 % confidence limit)

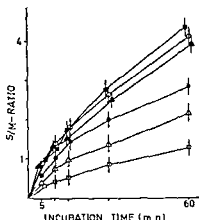


Fig. 3. Uptake of piribenzil by liver slices obtained from female rats, in the presence of some organic cations and anions. Each point is the mean of 4–12 experiments. Brackets indicate the S.D.

- ○ piribenzil 1 × 10^{-5} M
- □ piribenzil, 1 × 10^{-5} M + emepronium, 1 × 10^{-4} M
- △ piribenzil, 1 × 10^{-5} M + methylatropine, 1 × 10^{-4} M
- ● piribenzil, 1 × 10^{-5} M + xylocaine 1 × 10^{-4} M
- ■ piribenzil, 1 × 10^{-5} M + phenolphthalein glucuronide, 1 × 10^{-4} M
- ▲ piribenzil, 1 × 10^{-5} M + ampicillin, 1 × 10^{-4} M

Effect of some organic compounds known for extensive biliary excretion

The hepatic uptake of piribenzil in the presence of other organic compounds which are known to be eliminated in the bile to a large extent are shown in table 2 and fig 3 The quaternary ammonium compounds emepron bromide and methylatropine cause inhibition (P < 0 001) of the uptake of piribenzil Emepron bromide seems to be more effective than methylatropine Lidocaine which is a tertiary amine also causes a significant decrease (P < 0 001), but this compound seems to have a somewhat smaller effect than emepron bromide and methylatropine

The acidic compounds phenolphthalein, phenolphthalein glucuronide, BSP, and phenemal do not seem to influence the uptake process of piribenzil, nor do the two penicillins Azidocillin is an acid and ampicillin an ampholyte

Chromatography studies

Fig 4 shows the distribution of radioactivity after chromatography of ethanol extracts of homogenates from liver slices The chromatograms showed only one peak and this peak has the same Rf value as that of authentic piribenzil (Rf ~ 0 5) at all incubation times (5–60 min) The peak height increased with increasing incubation time

Binding to liver homogenate

Table 3 shows the binding of radioactivity to various dilutions of liver homogenate as revealed by ultrafiltration The concentration of piribenzil was the same in these experiments (10^{-5} M) but the dilution of liver homogenate varied from 3–31 % (W/W) The results indicate extensive binding

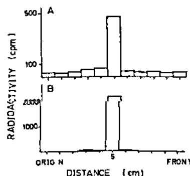


Fig 4 Thin layer chromatography of rat liver homogenate obtained from an uptake study with ^{3}H piribenzil The plate (silica gel) was developed with n butanol formic acid H O (4 1 2)
A 60 min incubation
B authentic ^{3}H piribenzil

Table 3

Binding of ^3H piribenzil to various dilutions of liver homogenate as revealed by ultra filtration The concentration of piribenzil was 10^{-5} M in all experiments Results are given as mean values from 5 experiments ± S D Dilution factor 0 03 means a 3 % (W/W) liver homogenate

Dilution factor	Bound piribenzil (%)
0 03	25 3 ± 2 0
0 07	34 3 ± 2 9
0 14	43 5 ± 1 6
0 31	60 1 ± 1 9

of piribenzil to liver component(s) At the liver dilution factor 0 03 about 25 % of the radioactivity was bound to the liver homogenate and at the liver dilution factor 0 31 about 60 % was bound

Efflux studies

The results showed (table 4) that the efflux of radioactivity was decreased when iodoacetic acid was present in the medium, as compared with the control samples Statistical significance was obtained after 20 and 40 minutes incubation ($P < 0$ 01)

Discussion

The present study suggests that the uptake of piribenzil into liver slices is partly energy dependent The metabolic inhibitors 2 4 dinitrophenol and iodoacetic acid significantly decreased the uptake of piribenzil ($P < 0$ 001)

Table 4

Efflux of radioactivity from rat liver slices pre incubated (60 minutes) with ^3H piri benzil in Tyrode s buffer The effect of iodoacetic acid (1×10^{-3} M, medium concen tration) was investigated Mean values ± S D are given from 5 experiments

Incubation time min	Sample	Radioactivity d p m /100 µl medium	P
10	control	8106 ± 796	N S*
	iodoacetic acid	8113 ± 346	
20	control	11263 ± 603	< 0 01
	iodoacetic acid	10140 ± 253	
40	control	13490 ± 886	< 0 01
	iodoacetic acid	11680 ± 416	

* N S not significant (at 95 % confidence limit)

Iodoacetic acid blocks glycolysis and reduces the supply of pyruvate for oxidative metabolism 2 4 dinitrophenol is an uncoupler of oxidative meta bolism Incubation in N_2-atmosphere also decreased the uptake ($P < 0.01$) The effect of these metabolic conditions leads to a decrease or cessation of the generation of ATP, required for e g energy dependent transport systems The fact that 2 4 dinitrophenol, iodoacetic acid and incubation in N_2-atmosphere decreased the uptake of the amino compounds PAEB (SOLOMON & SCHANKER 1963) and CGT (GIGON *et al* 1969) into liver slices support the results obtained in this study with piribenzil The effect of ouabain, a suggested inhibitor of Na^+, K^+-APT-ase, was not so evident Inhibition of the uptake was noted when the concentration of ouabain was 10^{-3} M but not when the concentration was 10^{-4} M Sodium cyanide was found to have no statistically significant effect on the uptake of piribenzil It is known that cyanide decreases the bile flow but does not influence the excretion of cholecophilic substances such as BSP and bilirubin (HARGREAVES & LATHE 1963, VANLERENBERGHE *et al* 1970) SOLOMON & SCHANKER (1963) found no effect of cyanide on PAEB uptake into liver slices

It should be remembered that these results do not make clear whether the observed uptake (S/M values) is confined to the intracellular space of the liver parenchyma and/or that of bile canaliculi A significant decrease in the uptake ($P < 0.05$) was noted after 10 minutes incubation with the metabolic inhibitors iodoacetic acid and 2 4-dinitrophenol After 5 minutes a decrease in the uptake was already noted, however, this decrease was not significant This may be due to the fact that a too short preincubation time (10 minutes) with inhibitor was used These results suggest an energy dependent uptake into the intracellular compartment The results from the secretion" studies showed that the efflux of piribenzil from the slices was decreased by iodoacetic acid This may be interpreted as an energy dependent transport system directed outwards from the slices However, this study does not give any information as to whether this process is localized to canalicular sites and/or to other sites of the liver parenchyma

Since the water content of liver slices is about 75 % of wet weight (SOLOMON & SCHANKER 1963) one would expect an S/M value of about 0 75 if a compound is distributed equally between the extracellular and intra cellular water In none of the experiments with metabolic inhibitors and incubation in N_2-atmosphere was the S/M value reduced to about 0 75 This may indicate the presence of other uptake mechanisms than an energy dependent transport system Such processes could be responsible for the binding of the compound to intracellular components or biotransformation, thus reducing the concentration of free substance in the cytosol Ultra-filtration studies showed that piribenzil was highly bound to liver homogenate Extensive binding of CGT to li

et al 1969) but PAEB showed almost no such binding (SOLOMON & SCHANKER 1963) Uptake due to rapid biotransformation does not seem to be of major significance since the chromatographic studies only indicated the presence of unchanged piribenzil in the slices

It is possible that the energy dependent uptake is a rapid process transferring the compound into the intracellular space and that binding to intra cellular components e g protein occurs afterwards This does not exclude the possibility that the observed binding involves membrane components

Emepron bromide and methylatropine which structurally are close to piri benzil, with an onium group at one end of the molecule and one or two non polar ring structures at the opposite end of the molecule, separated by an intermediate chain, significantly decreased the uptake of piribenzil ($P < 0\,001$) Lidocaine which is a tertiary amine also caused significant inhibition Emepron bromide which structurally is most close to piribenzil, was the agent most effective in blocking the uptake while lidocaine showed the least effect The studies may indicate that if drugs with similar molecular properties are given simultaneously to a patient they may interact during their excretion processes, a possibility which may be of clinical importance

It is often considered that organic anions and cations are secreted by separate transport systems, e g SCHANKER (1968) This seemed to be of relevance in this study with piribenzil Phenolphthalein, phenolphthalein glucuronide, BSP, and phenemal did not influence the uptake of piribenzil The two penicillins, ampicillin and azidocillin, too did not decrease piribenzil uptake All these compounds are anions (ampicillin is an ampholyte) and are known to be extensively excreted in the bile

The present results suggest that the uptake into liver slices of piribenzil is energy dependent and that binding to tissue components occurs Efflux from liver slices containing piribenzil, also seems to be energy dependent These *in vitro* experiments may reflect processes responsible for the biliary excretion of organic cations

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Inhibition of Free Fatty Acid Mobilization by Nicotinic Acid in Canine Subcutaneous Adipose Tissue *in Situ*: Combination of Lipolysis Inhibition and Increased Re-esterification

By

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Abstract Canine subcutaneous adipose tissue was isolated and auto perfused *in situ* Nicotinic acid (NicA) (4 or 40 mg/kg intravenously caused an increased lactate release and/or a decreased pyruvate release from the tissue Following the administration of the higher dose of NicA the glucose uptake was also enhanced While there was no inhibition of basal lipolysis basal FFA release was significantly reduced following the higher dose of NicA Lipolysis induced by sympathetic nerve stimulation (4 Hz for 10 min) was inhibited by 60–80 per cent while induced FFA release was inhibited by 90 per cent or more This increase in re esterification was statistically significant It is concluded that re-esterification plays an important role in the FFA lowering effect of NicA

Key words Nicotinic acid – adipose tissue – lipolysis – esterification

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Following the discovery by CARLSON & ORO (1962) that nicotinic acid (NicA) lowers plasma free fatty acid (FFA) levels and antagonizes the rise after noradrenaline infusion, several attempts to explain the mechanism of action have been made (cf GEY & CARLSON 1971) Studies on white adipose tissue *in vitro* have demonstrated that NicA interferes with the activation of lipolysis but has little effect on the lipase itself (CARLSON 1963, BJORNTORP 1965) Studies on isolated fat cells from the rat have indicated that there is an interference with the formation of cyclic AMP (BUTCHER *et al* 1968, WILLIAMS *et al* 1968) Some investigators have reported that the lipolytic action of exogenous cyclic AMP is also antagonized by NicA, indicating that inhibition also occurs at subsequent steps in the activation sequence (NAKANO 1970, HOLLMAN *et al* 1972), while others have failed to demonstrate such an inhibition (e g HEPP *et al* 1971) Furthermore, it has been

shown that NicA increases triglyceride synthesis *in vitro*, which might suggest that part of the FFA lowering effect *in vivo* is due to an increased re-esterification (LEE *et al* 1961, ÖSTMAN 1964, SOLYOM & PUGLISI 1966, HOLLMAN *et al* 1970)

Since these studies on the mechanism of action have all been performed *in vitro*, there is little information concerning the relative importance *in vivo* of lipolysis inhibition and re-esterification as means of lowering the plasma FFA levels In the present paper some findings related to this question will be reported The antagonism by intravenously administered NicA of glycerol and FFA release from canine subcutaneous adipose tissue was therefore studied It is assumed that glycerol release is an adequate measure of lipolysis (STEINBERG & VAUGHAN 1965) The results indicate that increased re-esterification, defined here as a greater depression of FFA release than of glycerol release, plays an important part in decreasing FFA mobilization from white adipose tissue *in situ*

Materials and methods

The experiments were conducted on fed female mongrel dogs weighing 7–15 kg Anaesthesia was induced by 25–30 mg/kg sodium pentobarbital (pentobarbitalum INN mebumalum NFN) and was supplemented by further single doses of 25–50 mg intra venously during the course of the experiment Subcutaneous adipose tissue in the inguinal region was isolated from all the surrounding tissues (ROSELL 1966) and the artery and vein leading to the preparation (weight 19–53 g) were cannulated with polyethylene catheters Heparin (Vitrum) was administered in a dose of approximately 2 500 i u /kg intravenously about two hours before the experiment to prevent clotting of the blood Blood was directed to the adipose tissue from the ipsilateral femoral artery via an exteriorized loop of plastic tubing into which a drop counter was in cluded to monitor the blood flow The venous blood was directed back to the general circulation via the ipsilateral femoral vein The blood flow and the blood pressure measured by a Statham P 23 AC transducer in the contralateral femoral artery were recorded on a Grass polygraph The nerve suppplying the tissue was cut at the level of the external hiatus of the inguinal canal and placed on a bipolar silver electrode protected from drying by Plastibase (Squibb) Electrical pulses of supramaximal in tensity (12–13 V) and duration (2 msec) were delivered at the rate of 4 cps

Arterial blood samples were drawn from the femoral artery and venous samples were taken by allowing the venous blood to flow freely into ice-cooled centrifuge tubes After the determination of lactate (HOHORST 1962) pyruvate (BUCHER *et al* 1962) and haematocrit (39 51 per cent) the blood was centrifuged and aliquots of plasma were taken for the determination of glycerol (LAURELL & TIBBLING 1966) FFA (TROUT *et al* 1960) and glucose (GLOX Reagent KABI Stockholm Sweden) The rate of uptake or release of these substances was calculated from the arterio venous concentration difference and blood (or plasma) flow Control arterial and venous blood samples were drawn at least 90 min after the administration of heparin The sympathetic nerve was then stimulated for 10 min During and after stimulation venous samples

were drawn Thirty min after the end of the stimulation period a control arterial blood sample was drawn and an infusion of nicotinic acid (Acidum nicotinum INN NFN) at a rate of 0 2 (5 experiments) or 2 (3 experiments) mg × kg $^{-1}$ × min^{-1} in the brachial vein was commenced and was continued for 20 min Starting at about 10 and 60 min after the end of the NicA infusion a similar series of arterial and venous samples were drawn The pattern of sampling is shown in fig 1

Results

Administration of 4 mg/kg NicA (5 dogs) did not change the mean arterial blood pressure, which averaged 132 mmHg (115–155 mmHg), but a ten times higher dose (3 dogs) caused a drop of 5–10 mmHg NicA did not significantly change either adipose tissue blood flow (average resting value 13 3 ± 2 4 ml × min $^{-1}$ × 100 g $^{-1}$) or the vasoconstrictor response to nerve stimulation (215 ± 53 per cent increase in vascular resistance)

The metabolic effects of NicA (4 mg/kg) are illustrated in fig 1 and

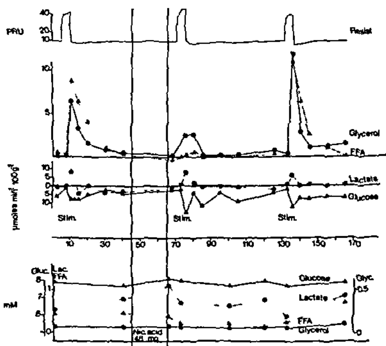


Fig 1 Effects of nicotinic acid infused at the rate of 0 2 mg × min^{-1} × kg^{-1} for 20 min via the brachial vein in one dog weighing 12 kg The isolated adipose tissue weighed 19 1 g and the haematocrit was 50–47 From top to bottom adipose tissue vascular resistance net release rate of FFA and glycerol rate of net release (+) or net uptake (−) of lactate and glucose in adipose tissue time (min) arterial concentration of glucose lactate FFA and glycerol

summarized in table 1 10–50 min after the NicA infusion the arterial FFA levels were decreased, while the glycerol and glucose remained unchanged There was no statistically significant change in the basal release or uptake of glycerol, FFA and glucose during this time interval On the other hand, the arterial lactate level and the rate of lactate release from adipose tissue was increased The net release of FFA following nerve stimulation (calculated as the net release during and 30 min after nerve stimulation minus the net release during the pre stimulatory control period for the same over all time) was completely inhibited, whereas the induced net release of glycerol was inhibited by about 60 per cent The difference in induced FFA and glycerol release, which indicates an increased re esterification, was significant at the five per cent level

Table 1

The effect of NicA (0 2 mg/kg infused intravenously for 20 min) in anaesthetized dogs The results are mean ± S E M from five experiments Statistical hypotheses were tested by a two-way analysis of variance

ARTERIAL CONCENTRATIONS

	Pre infusion of NicA (control) mM	10–50 min post infusion of NicA % change from control	60–110 min post infusion of NicA % change from control
Glycerol	05 ± 02	+10 ± 7	+127 ± 47 (P<0 05)
FFA	58 ± 21	−32 ± 8 (P<0 01)	+ 54 ± 42
Glucose	6 74 ± 88	+ 3 ± 2	+ 6 ± 3
Lactate	1 35 ± 26	+26 ± 7 (P<0 05)	+ 66 ± 30

ADIPOSE TISSUE UPTAKE (−) OR RELEASE (+)

	Pre infusion of NicA (control) μ moles × min^{-1} × 100 g^{-1}	10–50 min post infusion of NicA μ moles × min^{-1} × 100 g^{-1}	60–110 min post infusion of NicA μ moles × min^{-1} × 100 g^{-1}
Glycerol	20 ± 06	17 ± 03	44 ± 11 (P<0 05)
FFA	− 22 ± 28	08 ± 18	− 04 ± 09
Glucose	−4 15 ± 58	−4 37 ±1 64	−3 93 ±1 02
Lactate	13 ± 32	96 ± 49 (P<0 01)	67 ± 17

NET RELEASE FOLLOWING NERVE STIMULATION

	Pre infusion of NicA (control) μ moles × 100 g^{-1}	10–50 min post infusion of NicA % inhibition	60–110 min post infusion of NicA % inhibition
Glycerol	23 32 + 7.25	62 ± 8 (P<0 01)	45 ± 10 (P<0 01)
FFA	30 22 ± 10 75	107 ± 16 (P<0 01)	27 ± 17

Table 2

Effects of NicA (2 mg/kg infused intravenously for 20 min) in anaesthetized dogs The results are mean ± S E M from three experiments

ARTERIAL CONCENTRATION

	Pre infusion of NicA (control) mM	10–50 min post infusion of NicA per cent change	60–110 min post infusion of NicA per cent change
Glycerol	0 13 ±0 04	−40 ± 10 (P<0 05)	−20 ± 35
FFA	0 96 ± 14	−33 ± 8 (P<0 05)	−10 ± 46
Glucose	6 10 ± 42	+12 ± 24	−10 ± 25
Lactate	1 31 ± 12	+26 ± 5 (P<0 05)	+20 ± 18
Pyruvate	133± 010	− 8 ± 3	+ 4 ± 16

ADIPOSE TISSUE UPTAKE (−) OR RELEASE (+)

	Pre infusion of NicA (control) μ moles × min^{-1} × 100 g^{-1}	10–50 min post infusion of NicA μ moles × min^{-1} × 100 g^{-1}	60–110 min post infusion of NicA μ moles × min^{-1} × 100 g^{-1}
Glycerol	14 ± 01	27 ± 07	09 ± 01
FFA	10 ± 11	− 16 ± 05 (P<0 05)	00 ± 04
Glucose	−1 86 ± 92	−2 92 ± 86 (P<0 05)	−3 35 ±1 05 (P<0 05)
Lactate	78 ± 17	1 63 ± 12 (P<0 05)	1 32 ± 17 (P<0 05)
Pyruvate	065± 032	− 069± 042 (P<0 05)	0 34 ± 022 (P<0 05)

NET RELEASE FOLLOWING NERVE STIMULATION

	Pre infusion of NicA (control) μmoles × 100 g^{-1}	10–50 min post infusion of NicA per cent inhibition	60–110 min post infusion of NicA per cent inhibition
Glycerol	40 03 ± 8 92	−78 ± 5 (P<0 01)	−61 ± 4 (P<0 01)
FFA	47 32 ± 6 52	−90 ± 12 (P<0 01)	−95 ± 12 (P<0 01)

There was no significant change in the arterial level of FFA, glucose or lactate or in the net release from adipose tissue of these metabolites 60–110 min after NicA administration On the other hand, the arterial glycerol concentration and the net release rate of glycerol was increased, while the evoked glycerol and FFA release were still somewhat reduced during this period

The results of three experiments, in which a ten times higher dose of NicA was administered are presented in table 2 With this dose the pattern of metabolic changes was somewhat changed Not only the arterial FFA, but also the glycerol level was depressed during the first period following administration of NicA There was also a decrease in the basal rate of FFA

release, while the glycerol release remained unchanged and the glucose uptake increased The depression of stimulated FFA and glycerol release was of longer duration and there was no indication of a secondary rise in glycerol efflux In these experiments, pyruvate was also determined and the results indicate that pyruvate and lactate changed in opposite directions in the adipose tissue, indicating a more reduced state of the cytoplasmatic NADH/NAD system

Discussion

There was no evidence of inhibition of basal lipolysis at any time after NicA administration This is in agreement with *in vitro* findings which demonstrate that NicA does not inhibit basal lipolysis even when present in the incubation medium in very high concentrations (SCHWANDT *et al* 1967) Conversely there was a prolonged inhibition of lipolysis induced by sympathetic nerve stimulation This can be explained by the *in vitro* findings that NicA inhibits the activation of the lipolytic process rather than the lipase itself (see Introduction)

The increase in glycerol release following nerve stimulation is a reflection of increased lipolysis, as discussed by STEINBERG & VAUGHAN (1965) It is generally agreed that stimulation of lipolysis is caused by an activation of a hormone-sensitive triglyceride lipase, rather than by an activation of lower lipases, and one must therefore assume that three moles of fatty acids are formed per mole of glycerol (cf STEINBERG & VAUGHAN 1965) The finding that less than three moles of FFA are released per mole of glycerol following nerve stimulation therefore indicates that fatty acids are re utilized in the tissue to a considerable degree It has been found previously that in fed dogs, such as those used in the present study, glucose is likely to be the major substrate for oxidation and that oxidation of fatty acids can explain only a very minor fraction of the difference between the amount of fatty acids calculated to have been formed and the amount that is actually released (FREDHOLM & KARLSSON 1970) There seems to be little evidence to suggest that fatty acid oxidation is increased in adipose tissue following NicA, while there is considerable evidence that the process of fatty acid esterification is increased by the drug (LEE *et al* 1961, OSTMAN 1964, SOLYOM & PUGLISI 1966, HOLLMAN *et al* 1970) For these reasons the present finding that NicA caused a greater depression of stimulated FFA release than of stimulated glycerol release is taken to reflect an increased re esterification

The major source of the α-glycerolphosphate necessary for re-esterification of fatty acids in white adipose tissue of fed animals is commonly believed

to be glucose (see VAUGHAN & STEINBERG 1965) An increased uptake of glucose tends to increase re esterification in canine subcutaneous adipose tissue (FREDHOLM 1971b) Although NicA increases glucose uptake in adipose tissue *in vitro* (LEE *et al* 1961) it is unlikely that this is the major reason for the increase in re-esterification, since the lower dose of NicA did not produce an increased glucose uptake On the other hand, and increased release of lactate and/or a decreased release of pyruvate was a constant finding, indicating an increased cytoplasmic NADH/NAD ratio There is considerable evidence that in canine subcutaneous adipose tissue increases in this ratio are accompanied by an increased re-esterification (FREDHOLM 1970, 1971a, 1971b)

The present study has thus demonstrated that NicA causes an increased re esterification of fatty acids in adipose tissue *in situ* The complete inhibition of FFA mobilization that is occasionally seen following NicA is due to the combined effects of lipolysis inhibition and of increased re esterification

Acknowledgements

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Phenolic Metabolites in Urine and Faeces from Rats Given Radioactive ^{14}C-L-DOPA

By

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Abstract Urine and faeces of rats were investigated for phenolic compounds after oral administration of ^{14}C L DOPA *Germ free* and *conventional* rats were used The urinary radioactive metabolites detected were 3 methoxy-4 hydroxy phenylethylamine, 3 methoxy-4-hydroxyphenylacetic acid *m* hydroxyphenyl acetic acid and *m* hydroxyphenylpropionic acid *Conventional* rats excreted more radioactive *meta* compounds than the *germ free* rats The faeces of *germ free* rats contained predominately radioactive 3 methoxy 4 hydroxy phenylacetic acid and faeces of the *conventional* rats contained predominately *m*- and *p* hydroxyphenylacetic acid

Key words Dopa urine – faeces – rats

The metabolism of L-DOPA in rats have been extensively investigated (PELLERIN & D IORIO 1955, GOLDSTEIN & MUSACCHIO 1962, TYCE 1971, HORST & JESTER 1971, BAKKE 1971a & b, BORUD *et al* 1971) SANDLER *et al* (1969) suggested biotransformation of L-DOPA in man by intestinal microorganisms to dehydroxylated metabolites, and SMITH *et al* (1964), BOULTON & QUAN (1970) and BAKKE (1971a) found evidence for this in both guinea pigs and rats

BORUD *et al* (1971 & 1972) did not find a significant effect of intestinal microorganisms on L-DOPA metabolism in rats on a milk diet. To investigate rats on a *normal diet*, germ free and normal rats were given tracer doses of ^{14}C-L-DOPA, and radioactive metabolites from the urine, faeces and caecum content were detected

Materials and methods

Compounds and abbreviations

L-3(3,4 dihydroxyphenyl) alanine 3 C14 (14C-L-DOPA with a specific activity of 9 7 mci/mmol) was purchased from the Radiochemical Centre, Amersham, England Un labeled L DOPA (eldopar®) was a gift from Weiders Farmasøytiske A/S, Oslo, Norway N acetylated 3 methoxy-4-hydroxyphenylethylamine (N Ac MDA) was synthezized according to GOLDSTEIN *et al* (1961) 3 methoxy-4-hydroxyphenylalanine (vanyl alanine) 3,4-dihydroxyphenylethylamine (dopamine), 3 methoxy 4 hydroxyphenylethyl amine (MDA) 3 methoxy-4-hydroxy phenylacetic acid (VAA), 3,4 dihydroxyphenyl acetic acid (DHPAA), 3 methoxy 4-hydroxyphenylethanol (VE), 3 methoxy-4-hydroxyphenylethanolamine (NMN), 3 methoxy-4 hydroxyphenyl (N-methyl) ethanolamine (MN) 3 methoxy-4-hydroxyphenylformic acid (VFA) 3 methoxy-4-hydroxycinnamic acid (ferulic acid) *p* tyramine (pTA), *m* tyramine (mTA), *m* hydroxyphenylpropionic acid (mTPrA) *m* hydroxyphenylacetic acid (mTAA), *p* hydroxyphenylacetic acid (pTAA) *p* hydroxyphenylformic acid (pTFA), were obtained from commercial sources

Animal experiments

Adult rats of the CDF strain (*germ free* and *conventional* animals) were raised and reared as described elsewhere (MIDTVEDT & TRIPPESTAD 1970) All the rats received a pellet diet during these experiments, and they were starved 24 hours before the L-DOPA experiment The radioactive aminoacid (0.2 mg L-DOPA) was dissolved in 5 ml 50 % ethanol to which were added three drops of glacial acetic acid and 7.5 ml water before autoclaving at 120° for 20 minutes A solution of L-DOPA handled in the same way showed no decomposition The radioactive solution was divided into four portions, and these were given individually to two *germfree* and two *conventional* rats They consumed the L DOPA solution within two hours, and they were allowed free access to food and water The rats were placed in metabolic cages, and urine specimens were obtained by means of separators which disposed of the faeces The urines were collected at room temperature in sterile bottles from the *germ free* rats Urine from the *conventional* rats was collected in bottles containing 5 ml 1 N-HCl The bottles were changed every 24 hours and stored at −20° The faeces were collected at room temperature and stored at −20° Forty eight hours after L-DOPA was given to the animals they were killed by decapitation The digestive tract from the duodenum to the rectum was opened and the content was taken out and stored at −20° The walls of the duodenum, small intestine, caecum colon and rectum were washed with distilled water and fixed to filter paper and served as a plan of the digestive tract After drying the walls in the exsicators for several days, they were exposed to X ray film for two weeks

Chemical determinations

Creatinine in the urine was estimated according to Jaffe s method Hydrolysis of the urine was done at 37° for 24 hours at pH 5 5 with β glucuronidase + arylsulphatase (Helix pomatia, Calbiochem, U S A) after precipitating the sulphate with $BaCl_2$

The faeces, caecum content and pellet diet were vigorously shaken with 0 1 N HCl (pH 1–2) for two hours The suspensions were extracted with twice the volume of ethylacetate centrifuged, and the ethylacetate was carefully sucked of Extraction was repeated twice and the combined organic extracts were concentrated on a vacuum evaporator at 40°

Urinary aminoacids were concentrated on a Zeocarb 225H+ column, eluted with 10 %

ammonia solution and concentrated before electrophoresis and chromatography according to GJESSING (1963) Phenolic acids were detected after ethylacetate extraction by bidimentional paper chromatography according to ARMSTRONG *et al* (1956) Phenolic amines were concentrated on Dowex 50 Columns before paper chromatography according to KAKIMOTO & ARMSTRONG (1962) The spray reagent applied on the chromatograms for phenolic compounds was diazotized *p* nitroaniline (ARMSTRONG *et al* 1956)

Radioactivity on all chromatograms was detected with X ray film (Kodak Safety Film 3) followed by exposure for two weeks

Results

Urine

Phenolic compounds detected by diazotized *p*-nitroaniline in rat urine corresponding to 1 mg of creatinine, is shown in table 1. Twenty four and 48 hours urine samples from two *conventional* and two *germ-free* rats were collected The *meta*-compounds mTAA and mTPrA were the main spots on the phenolic acid chromatograms made from *conventional* rat urine *Germ-free rats* had pTAA as the main acid in urine and small amounts of the *meta*-compounds

Table 1 also shows which phenolic acids contained radioactivity after oral loads of ^{14}C-L DOPA Nearly all the radioactivity in the phenolic acid extracts was found in the first 24 hours urines The two *conventional* rats excreted radioactive VAA, mTAA, mTPrA and pTAA Small amounts of radioactivity also showed up on the X-ray films in the area corresponding to 3,4 dihydroxyphenylacetic acid The two *germ-free* rats excreted radioactive VAA, pTAA, mTAA and mTPrA Diazotized *p*-nitroaniline showed the typical colour of the *meta* compounds on the chromatograms different from the colour of the *para* compounds

A neutral radioactive spot appeared on the phenolic acid chromatograms made from hydrolysed urines, and the R_f values were similar to VE or N-Ac-MDA The amount of the neutral radioactive DOPA metabolites was similar for *germ free* and *conventional* rats

Phenolic amines in the urines contained radioactivity only in *MDA* as shown in table 1 There was no difference between *germ-free* and *conventional* rats NMN and pTA+mTA showed distinct spots on the chromatograms sprayed with diazotized *p* nitroaniline, but X ray film exposed for two weeks to the amines did not show any radioactivity in these amines

Free aminoacid analysis of the urines from all rats showed no radioactive DOPA or vanylalanine on chromatograms corresponding to 1 mg of creatinine However, in the urine of the *conventional* rats, radioactivity was found in three spots with small R_f values corresponding to conjugates of DOPA or dopamine The eluated conjugates from the chromatograms were not

Table 1

Phenolic compounds in rat urine after oral ^{14}C-L-DOPA

Compounds detected on chromatograms and X ray film	Abbreviation	2 conventional rats				2 germ free rats			
		DPNA*		X ray**		DPNA		X-ray	
		T***	F***	T	F	T	F	T	F
p hydroxyphenylacetic acid	pTAA	+	+	+	—	++	+	+	—
p hydroxyphenylformic acid	pTFA	+	+	—	—	+	(+)	—	—
m hydroxyphenylpropionic acid	mTPrA	++	+	++	—	+	+	+	+
m hydroxyphenylacetic acid	mTAA	++	+	++	+	+	+	+	—
m hydroxyphenylformic acid	mTFA	+	+	—	—	—	—	—	—
3 methoxy 4 hydroxyphenyllactic acid	VLA	—	—	—	—	—	—	—	—
3-methoxy-4-hydroxyphenylacetic acid	VAA	+	+	++	++	+	+	++	++
3 methoxy-4-hydroxyphenylformic acid	VFA	++	+	—	—	+	—	—	—
3 methoxy-4-hydroxycinnamic acid		++	+	—	—	++	+	—	—
3,4 dihydroxyphenylacetic acid	DAA	—	—	(+)	(+)	—	—	(+)	(+)
3 methoxy-4-hydroxyphenylethylamine	MDA	(+)		+		(+)		+	
3 methoxy 4 hydroxyphenylethanolamine	NMN	+		—		+		—	
3 methoxy-4-hydroxyphenyl(N methyl) ethanolamine	MN	(+)		—		(+)		—	
p tyramine + *m* tyramine	pTA+mTA	++		—		++		—	
3 methoxy-4-hydroxyphenylethanol + *N acetyl 3 methoxy-4-hydroxyphenylethylamine*	VE+ N Ac MDA	(+)	—	(+)	—	(+)	—	—	(+)

Phenolic acids and 3 methoxy 4-hydroxyphenylethanol (VE) + N-acetylated 3 methoxy-4-hydroxyphenylethylamine (N Ac MDA) were detected on bidimentional chromatograms made from extracts of urine containing 1 mg of creatinine Amines were detected on chromatograms after concentration on Dowex 50 columns DPNA* Diazotized *para* nitroaniline was used as spray reagent on the chromatograms X ray** films were exposed for two weeks to the chromatograms to make the radioactivity visible F*** Free acids and neutral VE + N Ac MDA were detected in urines without hydrolysis T*** Total amounts of the compounds (free + conjugated) were detected in the urines after enzymatic hydrolysis of urines with β glucuronidase + arylsulfatase at pH 5 5 for 24 hours at 37° Intensity of spots on the chromatograms or the radioautograms are indicated as (+) trace, + distinct spot and ++ strong spot

Table 2

Phenolic acids in caecum content and faeces from rats after oral ^{14}C-L-DOPA

Compounds detected on chromatograms and X ray films	Faeces				Caecum content			
	2 convent rats		2 germ free rats		2 convent rats		2 germ free rats	
	DPNA	X ray	DPNA	X ray	DPNA	X ray	DPNA	X ray
p hydroxyphenylacetic acid	+	(+)	(+)	—	(+)	—	++	—
p hydroxyphenylformic acid	+	—	(+)	—	(+)	—	(+)	—
m hydroxyphenylpropionic acid	++	—	—	—	++	—	(+)	—
m hydroxyphenylacetic acid	++	(+)	(+)	—	++	—	+	—
3 methoxy-4-hydroxyphenyllactic acid	—	—	—	—	—	—	—	—
3 methoxy-4-hydroxyphenylacetic acid	+	—	—	(+)	+	—	—	—
3 methoxy-4-hydroxycinnamic acid	+	—	—	—	+	—	—	—

Phenolic acids in faeces corresponding to half the daily output were extracted and the acids in caecum content corresponding to 4/5 of the content was extracted The concentrated extracts were applied on paper chromatograms and run bidimentional, exposed to X ray film (X ray) *for two weeks before spraying with diazotized p-nitroaniline* (DPNA) For Explanation of the table see table 1

hydrolysed by 1 N-HCl for 30 minutes at 100° *Germ free rats* also showed some radioactive compounds on the aminoacid chromatograms in the same "conjugate" area. Following hydrolysis in 1 N-HCl they split up into radioactive MDA and vanylalanine

Faeces Table 2 shows the phenolic acids found in the faeces from the rats The main acids in *conventional* rats were mTAA, mTPrA and smaller amounts of pTAA, pTFA and VAA Radioactivity was detected in pTAA and mTAA, but not in VAA *Germ-free* rats showed phenolic acids pTAA, mTAA and pTFA, but radioactivity was found only in VAA in their faeces

Gut wall The rats were killed 48 hours after they had consumed the L-DOPA, and the caecum wall as well as the walls of colon and rectum contained radioactivity Acidic extracts of caecum content did not show any radioactivity on the chromatograms as shown in table 2 *Conventional* rats had considerable amounts of mTAA and mTPrA with smaller amounts of pTAA, pRFA and VAA, whereas *germ-free* rats had pTAA as the main phenolic acid in their caecum content with smaller amounts of mTAA, mTPrA and pTFA

The pellet diet was extracted for phenolic acids, and we found pTAA, mTAA and several other unknown compounds on the chromatograms Some of these unknown compounds appeared in the urine as well as in the faeces and caecum content from the rats

Discussion

In spite of the few experiments with only four rats the results in table 1 are clear The urinary metabolites from oral tracer doses of L-DOPA were the dehydroxylated phenolic acids pTAA, mTAA and mTPrA which were found in similar amounts as VAA (see fig 1) These results are in agreement with the studies of BAKKE (1971a & b), but different from our earlier studies on rats on a controlled diet and given large doses of L-DOPA (BORUD *et al* 1972) *Conventional* rats metabolized a greater percentage of L-DOPA to the *meta* metabolites than the *germ free* rats But we also found distinct radioactive spots in the mTAA and mTPrA on the chromatograms made from the *germ-free* rat urines This was confirmed by diazotised *p*-nitroaniline The extraction of phenolic acids for the pellet diet showed that *germ free* rats can excrete exogenous mTAA and mTPrA from the diet, but the radioactive acids must originate from the ^{14}C-L-DOPA This is an interesting finding since the *meta* acids normally present in rat urines were expected to be of exogenous or bacterial origin (ARMSTRONG & SHAW 1956, SCHELINE & MIDTVEDT 1970, BAKKE 1971b) The earlier results from this laboratory also showed that *germ free* rats excreted small amounts of mTAA, but we did not find an increased excretion of this acid after the L DOPA

Fig 1 Metabolism of L DOPA in the rat Radioactivity in N acetylated amines phenolic alcohols and phenolic acids detected in urine and faeces after oral tracer doses of ^{14}C L-DOPA (L DOPA ˣ)

experiments (BORUD *et al* 1971) The results in table 1, however, support the findings of BAKKE (1971a) that *m*-hydroxyphenol compounds are excreted in urine of rats given L-DOPA

The dehydroxylation of the dihydroxyphenyl compounds in the rat occurs mainly in the *para* position (DE EDS *et al* 1957, SCHELINE *et al* 1960), but we also detected a significant amount of radioactive pTAA in the urines both from *conventional* and *germ-free* rats A significant metabolism of L-DOPA to pTAA was found and this is in agreement with the findings of BOULTON & QUAN (1970) They reported *p* tyramine as a metabolite in rat brain from DL-DOPA 2^{14}C injected, and thus showed that dehydroxylation in the *meta* position of DOPA occurs in rats

Urinary amines showed no radioactivity in the pTA or mTA, except for the MDA both in *conventional* and *germ free* rats This indicated that dehydroxylation to the *meta* and *para* hydroxyphenolic acids in our rats takes place after monoamine oxydase has deaminated dopamine as indicated in fig 1 BAKKE (1971b) suggested that the dehydroxylation occurs at the final acidic stage, and he found evidence for this in experiments with intestinal microorganisms from rats

In spite of a distinct spot of NMN on our amine chromatograms, no radioactivity was detected This indicates a very slow metabolism from the radioactive L-DOPA to noradrenaline and NMN in the rats, a finding which is in agreement with earlier reports of PELLERIN & D IORIO (1955), TYCE (1971), HORST & JESTER (1971)

Table 2 shows phenolic acids detected in the faeces and caecum content from the rats given radioactive L-DOPA The daily excretion of phenolic acids in rat faeces can be similar to the amounts found in urine, and this may be important when studying the excretion of metabolites from known precursors in the rat TYCE (1971) detected several metabolites from L-DOPA in the bile when investigating isolated perfused rat liver, and these compounds may be excreted both in the faeces and in the urine In table 2 it is also shown that the caecum content contained phenolic acids even in *germ-free* rats As in the urines the meta-compounds mTAA and mTPrA predominated in the faeces and caecum content from the *conventional* rats In the faeces from *germ-free* rats we could see traces of mTAA, pTAA and pTFA (dietary origin?), and in the caecum content pTAA was the main acid

Faeces from the *conventional* rats collected during the first 24 hours after oral 14C L-DOPA contained radioactive pTAA and mTAA but no VAA The two *germ free* rats only showed radioactivity in VAA in their faeces These findings indicate that the bacterial flora is involved in the metabolism of L-DOPA The walls of the digestive tract from the rats killed 48 hours after L-DOPA treatment contained radioactivity The caecum wall showed the strongest ray emitting area, and *germ free* and *conventional* rats showed the same intensity of radioactivity in the digestive tract The caecum content did not contain any ^{14}C phenolic acids or neutral compounds This indicates that the radioactivity in the walls of the digestive tract is probably due to bound dopamine

In conclution we found that *germ-free* and *conventional* rats excreted the same pattern of L-DOPA metabolites in urine All rats excreted the dehydroxylated metabolite pTAA *Conventional* rats excreted more mTAA and mTPrA than *germ free* rats Only MDA was detected as the amine metabolite from L-DOPA, and this indicates that the dehydroxylation of DOPA metabolites takes place at the late acidic stage

Faeces from *conventional* rats contained radioactive pTAA and mTAA, whereas faeces from *germ free* rats contained only radioactive VAA This indicates that microorganisms play a part in the dehydroxylation of L-DOPA metabolites in the rat

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The Distribution and Fate of ^{14}C-Cloforex in the Lung of the Rat

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Key words Cloforex – chlorphentermine – lung distribution

Whole-body autoradiography with labelled cloforex [ethyl N (2 (*p* chlorphenyl)-1,1-dimethyl) carbamate], an anorectic agent structurally related to amphetamine, has shown in mice a marked accumulation of radioactivity in the lung (RYRFELDT 1970) This finding may be correlated to the clinical observations of pulmonary hypertension obtained with some anorectic drugs of this type (GURTNER *et al* 1968a & b, LANG *et al* 1969, SCHWINGSCHACKL *et al* 1969, OSTERMAN & TEGNER 1969) Accumulation of foam cells in the alveoli of the lungs of rats has been found after cloforex administration (MAGNUSSON & MAGNUSSON 1972)

In order to study the distribution in the lung in more detail a micro-autoradiographic study was performed using labelled cloforex The only labelled substance available was ^{14}C-labelled in two methyl groups, which of course limits the autoradiographic resolution The labelled substance was obtained from the Radiochemical Centre, Amersham, England and had a specific radioactivity of 20 μci/mg To be able to study the distribution of radioactivity also in the foam cells, which develop after prolonged cloforex administration, 4 male Sprague Dawley rats were dosed orally with non radioactive cloforex (75 mg/kg) dissolved in Cremophor® El water solution (25 %, w/w) for 10 days Four control rats received vehicle solution only On the day of 11th one oral dose of ^{14}C-cloforex + carrier (200 μci/kg 75 mg/kg) was given to all the animals Four and 24 hours after administration 2 rats from each group were killed, the lungs dissected free and samples taken for 1) freeze drying and 2) total radioactivity measurement and extraction, in order to find out which substance(s) the radioactivity corresponded to

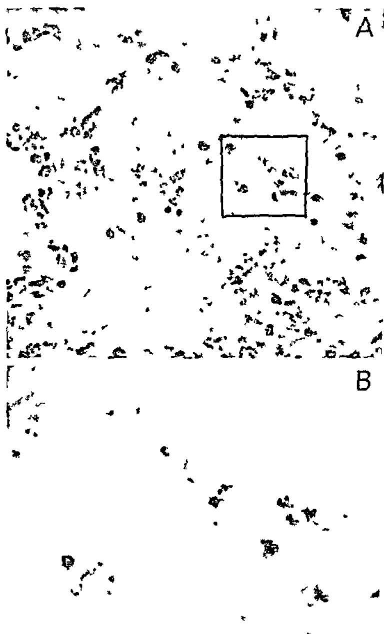


Fig 1A M cro-autoradiogram showing the d stribut on of rad oactiv ty in the lung of a rat dosed with [14]C-cloforex orally The rats was treated w th non rad oactive cloforex (75 mg/kg) orally for 10 days before the adm n stration of the labelled drug The rad o-activity is localized over the normal alveolar cells as well as over the foam cells (large cell in framed area see B) × 150 Fig 1B Framed area of A in higher magnifi at on × 700

After freeze drying the specimens were embedded in paraffin wax while still kept *in vacuo*, and micro-autoradiographed according to the "dry" method described by HAMMARSTROM *et al* (1965)

The radioactivity was localized over the normal alveolar cells as well as over foam cells, in the lung No radioactivity was seen over the alveolar space or in the bronchi (fig 1)

Total radioactivity measurements showed that in control animals the 24 hour value of total radioactivity (3 9 $\times$ 10^5 d p m /g lung tissue, mean, N=2) was higher than the 4 hour value (1 6$\times$$10^5$ d p m /g tissue) However, in pretreated animals the radioactivity was about the same at both times (3 8 $\times$ 10^5 d p m /g tissue)

The radioactivity in the lungs were assayed after homogenization of the tissue samples and then performing acid (pH $\sim$ 1) and alkaline (pH $\sim$ 12) extraction with methylene chloride Unchanged cloforex is obtained after acid extraction and chlorphentermine [1-(*p*-chlorophenyl)-2-methyl-2 propyl amine] a metabolite of cloforex, after alkaline extraction The separation capacity of the extraction procedures was checked by thin layer chromatography as described previously (RYRFELDT 1970) By analysis of simulated lung homogenate samples, it was established that the mean recovery of cloforex was 93 6 $\pm$ 0 7 % (mean $\pm$ S E M , n = 5) and the corresponding value for chlorphentermine was 98 9 $\pm$ 2 2 % (n = 8)

The results showed (table 1) that after one single oral administration of ^{14}C-cloforex the main part of the activity in the lung samples was attributable to ^{14}C-chlorphentermine Only minor amounts of unchanged drug were found The same was found in the pretreated animals

Table 1

Ratio of ^{14}C-cloforex and ^{14}C chlorphentermine to total radioactivity of lung samples after a single oral administration of ^{14}C cloforex to rats (75 mg/kg)

Pretreatment	Animal no	Time after administration of ^{14}C cloforex (hours)	% of total radioactivity cloforex	chlor phentermine
Control	1	4	7 8 $\pm$ 0 4*	85 3 $\pm$ 0 7
	2	4	5 0 $\pm$ 0 7	94 5 $\pm$ 1 1
	3	24	4 3 $\pm$ 0 5	94 8 $\pm$ 2 2
	4	24	4 9 $\pm$ 0 7	98 4 $\pm$ 3 2
Non radioactive	5	4	4 4 $\pm$ 0 8	86 6 $\pm$ 3 1
cloforex orally	6	4	3 5 $\pm$ 0 2	94 8 $\pm$ 1 7
for 10 days	7	24	5 3 $\pm$ 0 7	91 3 $\pm$ 3 3
(75 mg/kg)	8	24	6 0 $\pm$ 0 3	90 0 $\pm$ 3 9

* mean $\pm$ S E M (n=3)

Thus it is plausible to consider that a metabolite of cloforex, chlorphentermine, is the active substance causing the pathological lung changes observed in connection with cloforex administration to rats and that this metabolite is localized to normal cells as well as to foam cells

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Knud O. Møller

June 21, 1896 – August 23, 1973



As a son of a doctor, Knud O Møller naturally studied medicine He graduated in 1922 from the University of Copenhagen, and soon became assistant in the Pharmacological Institute where he kept this position except for an interval during the years 1926 – 28 when he received a throughout clinical training In the Pharmacological Institute his research was mainly concentrated round the effect of diuretics for which he received a Ph D in 1926 In 1938 he took over the chair in Pharmacology at the University of Copenhagen, at that time the only medical school in Denmark

As soon as he was established he devoted himself to teaching In an astonishing short time he single handed wrote the first modern textbook in pharmacology in Scandinavia He was not without educational training he had taught pharmacology at the College of Dentistry from 1927 and had also written a pharmacological textbook for dentists The first edition appeared in 1941, and its subtitle was "The rational basis of drug therapy' His clinical education allowed him to describe the mutual interaction between diseases and drugs so that his book almost became a textbook in *clinical pharmacology* It soon became very popular amongst physicians in general practice or with specialists in internal medicine Over a long period it was used as textbook in universities all over Scandinavia and its German translation was used in quite a few medical schools in Central Europe It ran through 6 editions and was revised according to the development of pharmacotherapy The latest edition appeared in 1966, but unfortunately the rapid development in the field made the book outdated in some respects, and the many textbooks in English of which new editions appeared at very short intervals partly replaced Møller's books in teaching, though it is still found on the shelves of many Scandinavian doctors and often used for references in cases of doubt Møller was easily readable, as his style was precise and the material well prepared, not only in his books, but also in his many review articles in which he informed the Danish medical profession about recent progress in pharmacology and about the proper use of drugs

Møller s predecesser had little interest in scientific work, but Møller rapidly opened his institute to young researchers, mostly with a medical background He supported their studies and research with enthusiasm with the result that many publications appeared from the Institute of Pharmacology of the University of Copenhagen Much of the work was done for practical purposes, sometimes in collaboration with clinicisians Møller started the development of anaesthesiology in Denmark, and the first Danish anaesthesiologists began their career in his institute Another achievement in which the Pharmacological Institute was involved was the treatment of patients poisened by hypnotics In collaboration with the clinicisians the institute developed a theoretical basis for the ' Scandinavian Method" in which the

treatment was directed against vascular shock, which brought down considerably the mortality of barbiturate poisoning

Since the establishment of the Pharmacological Institute of the University of Copenhagen, it was used by the police to supply information about the chemical and forensic examination of organs obtained from corpses of subjects suspected of dying from poisoning, also of materials suspected of being contaminated with poisonous substances To begin with this activity was on a modest scale, but successively the number of requests increased As a result Møller succeeded in establishing a toxicological institute with almost a dozen chemists and technicians engaged in the work The analytical methods were improved, made more sensitive, more specific and more exact These were published, not infrequently in connection with a report of interesting cases of poisoning Finally a special building was erected to house the Institute of Forensic Chemistry After Møller's retirement the main part of the analyses were taken over by the Institute of Forensic Medicine but the new institute gave a most wellcome grant for the enlargement of the Institute of Pharmacology which due to the tremendous increase in teaching and research was severely in need of space

It would be difficult to describe all the research activities in Møller's institute during the years The abovementioned important examples will suffice to show how great a part of the institute s research was conducted with direct practical aims Yet more basic research was also provided Amply grants from the *Danish Association against Rheumatic Diseases* allowed Møller to establish a laboratory for the study of connective tissue, its reaction to stimulation and the influence of drugs In his later years most of the experimental work of the institute was devoted to problems concerning connective tissue

Unfortunately, there was practically no permanent positions for professional pharmacologists in Denmark during the time in which Møller worked, either in universities or in industry This was perhaps the reason why Møller never founded a school in the classical sense With very few exceptions, his outstanding collaborators and pupils became scattered around with leading positions in other professions, such as internal medicine, clinical chemistry, *anaesthesiology, dermatology, pharmacy etc On the other* hand their basic education made some of them occasional, though useful advisers in various governmental institutions and commissions

By tradition Møller became the chairman of the Danish Pharmacopoiea Commission He took up this work with ardent energy, first in Denmark and later as a member of the common Scandinavian Pharmacopoiea Commission. Undoubtedly no small part of that essential work was influenced and spired by him, although in later years he withdrew more and this special work One subject was of particular interest to

naming of drugs His aim was to systematize the generic names by using well defined suffixes and prefixes in such a way that it would be easy to recognize the type and effect of a drug merely by its name Some of the names are accepted only by the Scandinavian Pharmacopoiea, such as phenemal for phenobarbital, resp phenobarbitone and diemal for barbital, resp barbitone Many of his other invented names are more widely used, and his activities made him a member of the WHO Nomenclature Committee

The story of this journal, Acta pharmacologica et toxicologica, has frequently been told (JACOBSEN 1956) and there is no need to go into all the details here At the end of 1943 during World War II with the German occupation of Denmark, the publisher Einar Munksgaard approached Møller and myself, at that time the only two pharmacologists in Denmark in leading positions, with the proposal of starting a Scandinavian journal of pharmacology The idea was not new to us, and had frequently been discussed between us A Scandinavian Pharmacological Society was founded in order to continue the existence of pharmacology as a profession and to secure the ownership of the new journal, and the first issue of Acta pharmacologica et toxicologica appeared in 1945 on the very day on which the war ended in Denmark Very naturally Møller became the editor in chief of the journal His editorial work was carefully prepared in every detail with the same sense of system and order with which he ran his institute, and for almost 20 years he scrupulously edited every manuscript submitted to the journal His spirit still influences the journal and the results speak for themselves

His vast experience made him the principal national authority on pharmacology Naturally he presided in several scientific societies, such as the Danish Pharmacological Society, the Scandinavian Pharmacological Society, the Society for Theoretical and Practical Therapy etc Many of the societies acknowledged their gratitudes to him by nominating him as honorary member These included the Danish Anaesthesiological Society, the Danish Pharmacological Society and the Scandinavian Pharmacological Society Officially in Denmark no commission committee or assembly, even with the slightest connection with pharmacological problems appeared without inviting him to take the chair In this way he remained an authority on the development in the field in Denmark for nearly three decades

His interests were far reaching He enjoyed literature and music, while sport for him was more than a hobby He was the co founder and the chairman for many years of the Danish Students' Sporting Association, and in his younger days he was seen rowing on the Sound every morning from early spring to late autumn

Møller will be remembered as being extremely helpful, and very few asked him for help without leaving him with at least some good advice, but he never compromised He formed his opinion and promoted or defended

it eagerly sometimes almost passionately On the other hand he was perfectly willing to modify or even change this opinion if faced with new facts which would convince him

He was a faithful friend and liberally opened his home to guests from all parts of the world He also enjoyed arranging congresses, attending meetings as a chairman or being in the audience, and sitting over drinks discussing problems, scientific or non-scientific, with his friends His birthdays which fell on the longest day of the year were legendary

Weakness forced him to slow down his activities in his later years, although he admitted it only with difficulty A few years after his retirement he became bedridden and disease prevented him from following the rapid development of the science to which he had devoted his life

Erik Jacobsen

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The Toxicity of Chlorpromazine and Mescaline on Mouse Cerebellum and Fibroblast Cells in Culture*

By

I F Roubein, M Samuelly+ and W Keup++

(Received January 8, 1973 Accepted April 11, 1973)

Abstract The toxicity of chlorpromazine (CPZ) and mescaline (MCL) to mouse cerebellum and fibroblast cells was studied *in vitro* CPZ proved to be more toxic than MCL to both tissues Fibroblast celles are more sensitive than the cerebellum to both CPZ and MCL

Key words Cerebellum – fibroblast cells – chlorpromazine – mescaline

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The toxic effect of chlorpromazine (CPZ) on the cerebellum in culture has been studied and described by NAKAZAWA (1960), but its lethal dose level on the cerebellum has not been reported Nor has a comparison been made between its effects on the cerebellum and on fibroblast cells of the same species

Relatively little is known regarding the toxicity of mescaline (MCL) on the central nervous system in culture In view of these facts, it seemed both logical and expedient that the cytotoxic and lethal dose levels of these two compounds be investigated, the objective being to assess their toxic potential on the cerebellum and on fibroblast cells obtained from the same species of animal

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Materials and methods

The cerebellum was removed from newborn Swiss Webster mice, after this the meninges and choroid plexus were stripped off, it was then sectioned into 8 parts Two explants were placed on a collagen-coated coverslip, fed with one drop (0 04-0 05 ml) of a nutrient medium containing no antibiotics and similar to that reported by ALLERAND & MURRAY (1968), it was maintained in a lying-drop position in Maximow assembly at 34-35° according to the method of BORNSTEIN & MURRAY (1958)

One litter of Swiss Webster mice embryos which had been obtained under aseptic conditions 2 to 3 days before birth, was used as the source of the fibroblasts used in this study The fibroblasts were prepared by trypsinization of the entire batch of minced embryos and this was followed by the separation of the cells by centrifugation The cells were grown as a monolayer in T flasks at 37° with Eagle's basal medium supplemented by 20 % calf serum, dihydrostreptomycin (0 2 mg/ml) and penicillin (200 i u /ml) The cultures were made periodically since cells that had not been subcultured more than 4 times were exclusively used in this study

Drug solutions for the cerebellum were prepared by dissolving CPZ (or MCL) in a mixture of equal amounts of Eagle s minimal essential medium and Earle s balanced salt solution The mixture was sterilized by filtration, after which the required amounts of the other nutrient medium constituents were added For the fibroblasts, the solutions were made by dissolving each of the drugs in a Mg and Ca free balanced salt solution (BSS) which was added to the feeding medium

A total of 143 cerebellum cultures were exposed to the drugs from the time of explantation, 54 cultures for MCL and 89 for CPZ. As controls, 117 cultures which were explanted from same cerebellum in each experiment were used, all cultures were examined 48 hours following explantation In determining the toxic effects of both drugs on fibroblast cells each dose level was studied in triplicate The drug solutions were added 24 hours after subculturing and the cultures were examined after 72 hours

Observations The cultures of the cerebellum were examined by bright field microscopy with a X40 fluorite, 1 mm working distance oil immersion lens The cytotoxicity level was determined by using the criteria of NAKAZAWA (1960) vacuolization withdrawal of processes cytoplasmic bubbling and granulation Cell death was considered to have occurred if growth had halted the explant had detached from the collagen, and the pH had remained unchanged

The fibroblast cells were essentially graded according to the recommendation of TOPLIN (1959) The criteria for cytotoxicity were changes in morphological appearance,

Results and Discussion

In the literature a wide diversity of cellular effects of CPZ has been described GUTZ & SPIRTES (1964) suggested that CPZ acted primarily on th cell membrane DUJOVNL & ZIMMERMANN (1968) reported that CPZ leakage of enzymes from cells in suspension Similar effects were

Table 1

Minimum cytotoxic and lethal dose levels (μg/ml) of mescaline and chlorpromazine for cerebellum and fibroblast cells

Tissue	*Mescaline* cytotoxic dose level	*Mescaline* lethal dose level	*Chlorpromazine* cytotoxic dose level	*Chlorpromazine* lethal dose point
Cerebellum	Minimum 500	2000–2500	Minimum 25	175–200
Fibroblasts	200–300	Minimum 600	20–30	Minimum 80

by ZIMMERMAN & KENDLER (1970) when Chang human liver cells were exposed to CPZ or to other phenothiazine derivatives They assumed that these drugs produced damage to the cells or altered the permeability of their membranes

Data in table 1 shows that CPZ is more toxic to the cerebellum and to fibroblast cells than is MCL The difference in cytotoxic dose levels of CPZ for both tissues was insignificant, but its lethal dose varied significantly, the minimum dose required for the fibroblast cells was 80 μg/ml whereas for the cerebellum it was 175–200 μg/ml Moreover, the same table indicates that the cytotoxic dose level of MCL for the cerebellum was almost twice that required to cause similar effects in the fibroblast cells The lethal dose level for MCL on the cerebellum was 3 to 4 times greater than the level required for the fibroblast cells The higher resistance of the cerebellum to both CPZ and MCL is noteworthy However, it must be taken into consideration that in these *in vitro* studies the cerebellum and the fibroblast cells were cultivated under different conditions, and this factor could have some effect upon the comparative resistance of the two tissue types to CPZ and MCL

Our finding that CPZ is more toxic to both tissues than MCL is in agreement with the *in vivo* studies of CAMPBELL & RICHTER (1967) and FRIEDMAN *et al* (1963) They found the LD50 in mice to be 400–800 mg/kg for MCL and 220 mg/kg for CPZ

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Modification by L-DOPA Methylester (H 19/61) of Amphetamine-Induced Brain Catecholamine Changes, Thermal Responses and Toxicity in Developing Mice

By

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Abstract The effect of 150 mg/kg of L DOPA methylester on brain catechol amines was studied in adult and developing mice aged 3–5 days and 13–15 days Noradrenaline was moderately increased in the developing brain but dopamine showed a manyfold increase The increase of dopamine was less pronounced in adult brain High doses of dl amphetamine (50–160 mg/kg) injected to L-DOPA pretreated mice further increased the dopamine levels in the developing brain The adult brain catecholamines were remarkably depleted provided the observation period was 4 hours after amphetamine Amphetamine lowered the body temperature in mice aged 13–15 days the fall was dose-dependent and potentiated by pretreatment with L DOPA In adult mice amphetamine caused hyperthermia which was not modified by L DOPA L-DOPA did not change the mortality rates induced by amphetamine in developing mice but infant mice pretreated with L DOPA showed a lower brain amphetamine concentration after death than the mice given amphetamine only In adult mice L-DOPA enhanced amphetamine toxicity Special attention is paid to the importance of different turnover rates of catecholamines during development as well as to the complex action of amphetamine on enzymes regulating catecholamine turnover It is assumed that the hypothermia of developing mice after amphetamine might result from the primary effect of amphetamine on dopaminergic mechanisms

Key words Age amphetamine – catecholamines – L DOPA

Several studies (Weissman *et al* 1966, Randrup & Munkvad 1966, Scheel-Kruger 1971) indicate that the central effect of amphetamine depends on a continuous synthesis of catecholamines (CA) in the brain Previous studies from this laboratory suggest that the relative resistance to amphetamine toxicity observed in developing mice could be, at least partially, due to the primarily low central CA level in developing mice and that

the mechanisms of amphetamine toxicity and CA-responses induced by the drug in adult and developing mice is a very complex process (ALHAVA 1972, ALHAVA & KLINGE 1972, ALHAVA 1973) The protective effect of inhibition of the CA biosynthesis by methyltyrosine against amphetamine toxicity in adult mice was well documented by MENON & DANDIYA (1967), and this could also be reproduced in developing mice (ALHAVA 1973) Amphetamine enhanced the central CA reduction induced by methyltyrosine in adult and developing mice, which was assumed to reflect the accelerating effect of amphetamine on CA metabolism (ALHAVA 1973)

On the other hand, the typical stimulation induced by amphetamine is inhibited by methyltyrosine and restored by the administration of L-DOPA, and amphetamine and L DOPA are known to potentiate each other (RANDRUP & MUNKVAD 1966, HANSON 1967) Furthermore, amphetamine-induced hyperthermia has been suggested as a lethal factor in amphetamine intoxication (MOORE 1963 & 1964), and in rats and rabbits the hyperthermia can be inhibited by blocking dopamine receptors (HILL & HORITA 1971, MATSUMOTO & GRIFFIN 1971) There is also some evidence about the importance of dopaminergic mechanisms in temperature control in mice (FUXE & SJOQVIST 1972)

In addition to the functional and biochemical immaturity of the central nervous system (CNS) of developing mice (KOBAYASHI *et al* 1963, AGRAWAL *et al* 1968), one possible explanation for the increased tolerance to amphetamine in developing mice might be the low level and slow development of the peak of brain amphetamine concentration observed in infant mice (ALHAVA & MATTILA 1972) Therefore, the aim of the present study was to analyze further the mechanisms of amphetamine toxicity in developing mice by investigating the effect of L-DOPA on amphetamine toxicity and the changes in the central CA levels as well as on amphetamine-induced thermal responses and tissue amphetamine concentrations

Materials and methods

General technique and environmental conditions

Adult male NMRI strain mice weighing 19–25 g and developing mice of either sex in age groups of 3 5 days and 13–15 days (body weights 1 9–4 0 g and 5 2–8 1 g) were kept under standard laboratory conditions (ALHAVA & KLINGE 1972) the litters kept with their mothers until used

The drugs used were L-DOPA methylester (H 19/61 Kistner Lab Gothenburg Sweden) and dl amphetamine sulphate The brain concentration of CA body temperature and tissue amphetamine content were determined after various treatments.

In all experiments L-DOPA methylester was dissolved in saline and the dose to the methylester Dl amphetamine sulphate was injected in aqueous so the doses refer to the base All the injections were made intraperitoneally ...

volume of 0.1 ml/10 g of body weight. The injection technique used for animals weighing less than 10 g has been previously described (ALHAVA 1972). After the injections the mice were put into opaque plastic cages (15 × 20 × 13 cm) in groups of 4 without access to food or water. In the thermal experiments the control mice were also fasting. The room temperature during the experiments was 24–25°. The values of noradrenaline (NA) and dopamine (DA) as well as that of tissue amphetamine are expressed as µg/g of fresh tissue. Student's t test was used for the statistical treatment of the results.

The assay of CA

The mice were injected at 9 a.m. with 150 mg/kg of L-DOPA methylester. The animals were decapitated 30 or 60 minutes after the injection, and the CA content of the whole brains was assayed.

Mice pretreated with H 19/61 30 or 60 minutes earlier were injected with high doses of amphetamine (50–160 mg/kg). They were decapitated immediately after spontaneous death or after the observation period of 1 or 4 hours. The whole brains were assayed for their NA and DA contents by the spectrophotofluorometric methods described by BERTLER *et al.* (1958) and CARLSSON & WALDECK (1958). The methods were slightly modified as previously described (ALHAVA & KLINGE 1972). The adult brains were analyzed individually. In developing mice each sample consisted of 4 pooled brains. The recovery of NA was 66.4 ± 2.7 % (mean ± S.E.M.) and that of DA 77.3 ± 2.8 %. The results were corrected for the respective recoveries.

Since L-DOPA methylester is known to be oxidized by both the ferricyanide procedure used for NA and the iodine procedure used for DA and eluted in the DA fraction, half of the DA eluate was oxidized with ferricyanide, which made possible to calculate the percentages of methylester of the actual DA values (HANSON & UTLEY 1965). At the age of 3–5 days the methylester contributed 7.7 ± 1.5 % (mean ± S.E.M.) to the DA values 30 minutes after the administration and 2.2 ± 0.5 % 60 minutes after the administration. At the age of 13–15 days the respective value for the methylester was 2.7 ± 0.7 % of the DA values both 30 and 60 minutes after the administration, and in adult mice 13.4 ± 2.4 % of the DA values including both periods after the administration. The DA values were corrected for the respective mean methylester concentrations, unless the experiment had continued for 4 hours after amphetamine injection.

Rectal temperature of mice

The rectal temperature of adult and developing mice of both age groups was measured with Ellab thermometer type TE 3. The infant mice were injected with 120 mg/kg of amphetamine, the 13–15 day old mice with 50 and 100 mg/kg and the adults with 50 mg/kg of amphetamine, after which the measurements were made every 15th minute during the first hour and then every 30th minute during the subsequent 3 hours. The effect of an intraperitoneal injection of 150 mg/kg of L-DOPA methyl ester, as well as the effect of L-DOPA methylester pretreatment, given 30 minutes before injecting amphetamine in the doses mentioned above, on the rectal temperature in all age groups of mice was studied, the intervals between measurements after amphetamine injection being as described above. The youngest mice were handled with forceps during the measurements.

Toxicity experiments and tissue amphetamine content

The effect of pretreatment with 150 mg/kg of L-DOPA methylester on the amphetamine toxicity in adult and developing mice was studied. Thirty minutes after the in-

jection of H 19/61 the 3 5-day-old mice were given 120 mg/kg of amphetamine The respective doses of amphetamine were 100 mg/kg to 13–15 day-old mice and 60 mg/kg to adult mice After amphetamine induced deaths the brains of the infant and adult mice and the brains and hearts of 13–15 day old mice were dissected The tissues concentration of amphetamine was measured spectrophotometrically as described by AXELROD (1954) The survivors were decapitated 4 hours after the amphetamine injection and the tissue content of amphetamine was similarly determined In all age groups the tissues of mice which died spontaneously after amphetamine were analyzed individually In determinations performed on the tissues of 4 hour survivors each sample consisted of 2 pooled brains or hearts except in infant mice in which these samples consisted of 3 brains The recovery of amphetamine from the brain was 87–90 % and from the heart 103 %

In order to study the possible interference by H 19/61 with the amphetamine assay, experiments with H 19/61 and amphetamine *in vivo* and *in vitro* were performed in adult brain homogenates H 19/61 did not change the recovery of amphetamine *in vivo* nor did it produce any colour reaction when given alone However, in experiments performed *in vitro* the presence of H 19/61 caused a slight decrease (5–10 %) in amphetamine recovery This was considered to be within the confidence limits of the method

Results

Effect of L-DOPA on the brain CA

As seen in table 1, 150 mg/kg of H 19/61 greatly increased the brain DA level in the youngest mice both at 30 and 60 minutes after the injection, while the increase in the NA level was only slight or absent In the older age group H 19/61 elevated the DA level even more, and now the NA level was also significantly increased In adult mice the changes in the CA content were very slight

Modification by L-DOPA of the amphetamine induced brain CA changes

Table 2 shows the effect of amphetamine on the CA level of infant mice after pretreatment with H 19/61 The most pronounced increase in the CA was seen in mice given 120 mg/kg of amphetamine 30 minutes after the pretreatment and decapitated 60 minutes later When the animals were killed 4 hours after the amphetamine injection the DA level was still 14 times higher than the control level while NA was only slightly increased A very high dose of amphetamine (160 mg/kg) increased the CA levels in mice decapitated 1 hour after amphetamine

Table 3 shows that in mice aged 13–15 days, 100 mg/kg of amphetamine killed some animals within 5 minutes irrespective of the interval between L-DOPA and amphetamine The DA content was markedly and the NA content moderately increased

It emerges from table 4 that the CA changes in response to amphetamine of adult mice pretreated with H 19/61 differed from those of the

Table 1.

Effect of 150 mg/kg of L-DOPA methylester (H 19/61) on the brain levels of NA and DA 30 and 60 minutes after the administration in developing and adult mice The number of experiments *is given in brackets*

Age (days)	Time after administration (min)	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
3–5	30	0 17±0 01 (10)	106	2 08±0 32*** (10)	1155
	60	0 19±0 01** (11)	119	2 76±0 15*** (11)	1559
	control	0 16±0 01 (6)	100	0 18±0 03 (6)	100
13–15	30	0 30±0 03* (9)	150	5 10±0.28*** (9)	2259
	60	0 31±0 04* (9)	155	3 31±0 41*** (9)	1379
	control	0 20±0 02 (6)	100	0 24±0 02 (6)	100
Adult	30	0 47±0 01 (10)	109	1 75±0 15*** (10)	194
	60	0 45±0 03 (9)	105	1 06±0 09 (9)	118
	control	0 43±0 01 (10)	100	0 90±0 05 (10)	100

* $P < 0\ 05$ difference versus control level
** $P < 0\ 01$
*** $P < 0\ 001$

Table 2.

Effect of 120 and 160 mg/kg of amphetamine on the brain NA and DA levels in H 19/61 pretreated mice aged 3–5 days The intervals between the two injections were 30 or 60 minutes and the time of decapitation *after amphetamine* 60 or 240 minutes The number of experiments is given in brackets

Dose of H 19/61 (mg/kg)	*Dose of* amphetamine (mg/kg)	Time after administration (*min*)	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
150	–	30	0 17 ± 0 01 (10)	106	2 08 ± 0 32*** (10)	1155
150	–	60	0 19 ± 0 01** (11)	119	2 76 ± 0 15*** (11)	1559
150	120	30+60	0 27 ± 0 03** (5)	169	8 01 ± 0 57*** (5)	4500
150	120	30+240	0 19 ± 0 02 (2)	119	2 46 ± 0 70*** (2)	1361
150	120	60+60	0 26 ± 0 02*** (5)	163	5 04 ± 1 04*** (5)	2800
150	160	30+60	0.24 ± 0 01*** (4)	150	7 09 ± 0 86*** (4)	3938
control	–	–	0 16 ± 0 01 (6)	100	0 18 ± 0 03 (6)	100

* $P < 0\ 05$ difference versus control level
* $P < 0\ 01$
* $P < 0\ 001$

Table 3

Effect of amphetamine on the brain NA and DA levels of H 19/61 pretreated mice aged 13–15 days The number of experiments is given in brackets

Dose of H 19/61 (mg/kg)	Dose of amphetamine (mg/kg)	Time after administration (min)	NA μg/g mean ± S E M	% of control level	DA μg/g mean ± S E M	% of control level
150	-	30	0 30±0 03* (9)	150	5 10±0 28*** (9)	2259
150	-	60	0 31±0 04* (9)	155	3 31±0 41*** (9)	1379
150	100	30+5 to 60	0 24±0 01 (8)	120	6 84±0 56*** (8)	2850
150	100	60+5 to 60	0 25±0 03 (6)	125	5 33±0 25 (6)	2220
control	-	-	*0 20±0 02* (6)	*100*	*0 24±0 02* (6)	*100*

* $P < 0\ 05$ difference versus control level
*** $P < 0\ 001$

Table 4

Effect of amphetamine on the brain NA and DA levels of adult mice pretreated with H 19/61 The number of experiments is given in brackets

Dose of H 19/61 (mg/kg)	Dose of amphetamine (mg/kg)	Time after administration (min)	NA µg/g mean ± S E M	% of control level	DA µg/g mean ± S E M	% of control level
150	–	30	0 47±0 01 (10)	109	1 75±0 15*** (10)	194
150	–	60	0 45±0 03 (9)	105	1 06±0 09 (9)	118
150	50	30+240	0 13±0 02*** (5)	30	0 13±0 03*** (5)	14
150	100	30+10 to 35	0 29±0 01*** (4)	67	2 47±0 05*** (4)	274
control	–	–	0 43±0 01 (10)	100	0 90±0 05 (10)	100

*** $P < 0\ 001$ difference versus control level

mice Mice decapitated 4 hours after 50 mg/kg of amphetamine showed a definite depletion of both NA and DA levels in contrast to the massive increase of DA seen in infant mice 4 hours after 120 mg/kg of amphetamine (table 2) A high dose of amphetamine (100 mg/kg) killed the animals within 35 minutes, at which time NA was decreased to 67 %, whereas the DA level was almost three times as high as the control level

Effect of amphetamine and L-DOPA on body temperature

It is seen from fig 1 that *in the youngest mice* the rectal temperature in the control group spontaneously decreased by about 5° during the 4 hour measurement period ($P < 0\,001$) Amphetamine (120 mg/kg) induced a similar decrease during the first 90 minutes ($P < 0\,001$), after which there was an increase of 1° during the subsequent 90 minutes ($P < 0\,02$) H 19/61 alone produced a temperature curve of about the same shape and level as 120 mg/kg of amphetamine, and the curve did not noticeably change when the mice were pretreated with H 19/61 before injecting amphetamine

From fig 2 it is seen that *13-15-day-old mice* had a higher and more stable control level of body temperature than infant mice The injection of 50 mg/kg of amphetamine caused a rapid decrease of about 3° in body temperature, which gradually returned to the control level during the 4 hour

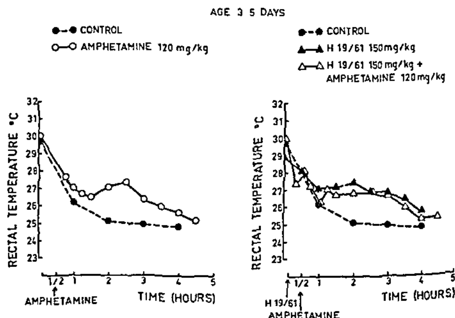


Fig 1 Body temperature responses induced by amphetamine and L-DOPA in mice aged 3-5 days Each point of the curves represents the mean ± S. E M of 10 animals

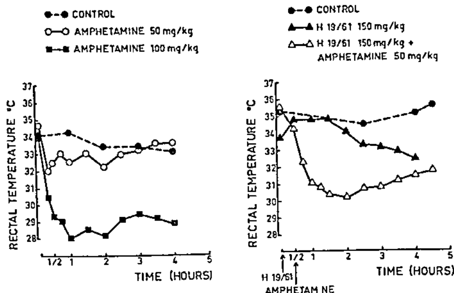


Fig 2. Body temperature responses induced by amphetamine and L-DOPA in mice aged 13–15 days Each point represents the mean ± S E M of 6 animals

period The hypothermic effect of amphetamine in this age group was dose dependent, since 100 mg/kg of amphetamine induced a twofold (6°) decrease in temperature as compared with 50 mg/kg The level of the curve induced by the larger dose remained low during the whole measurement period H 19/61 initially slightly increased (by 1°) the temperature during the first 90 minutes after which the temperature gradually decreased by about 2° in the remaining time period Pretreatment with H 19/61 enhanced the hypothermic effect of 50 mg/kg of amphetamine

In adult mice (fig 3) 50 mg/kg of amphetamine caused a typical hyper thermic response, which had completely subsided 5 hours later After H 19/61 there was an initial decrease (about 2°) in temperature during the first hour, and thereafter the temperature fluctuated between 36° and 37° The control level of adult body temperature was higher than either of those of the developing mice Pretreatment with H 19/61 did not prevent, nor potentiate, the hyperthermia induced by 50 mg/kg of amphetamine

Effect of L-DOPA on amphetamine toxicity and tissue content

In mice aged 3–5 days pretreatment with H 19/61 did not change the mortality rate induced by 120 mg/kg of amphetamine the mortality in th pretreated group was 9/16 and in mice injected with amphetamine al

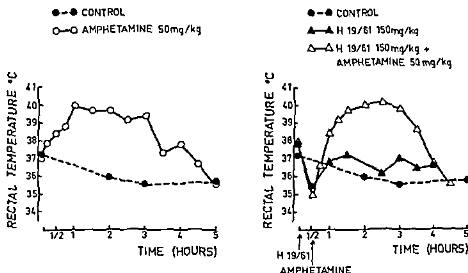


Fig 3 Body temperature responses to amphetamine and L-DOPA in adult mice Each point represents the mean ± S E M of 6 animals

7/12 However, table 5 shows that pretreated mice which died within 15 minutes after amphetamine had about a 50 % lower brain amphetamine concentration than the unpretreated mice ($P < 0\,001$) The survivors decapitated 4 hours after amphetamine showed the same brain amphetamine level in both groups

Table 5

Effect of pretreatment with L-DOPA methylester on amphetamine toxicity and brain amphetamine concentration in mice aged 3–5 days The number of experiments is given in brackets

Pretreatment with H 19/61 (mg/kg)	Dose of amphetamine (mg/kg)	Time of death after amphetamine	Brain amphetamine (μg/g) mean ± S E M
150	120	15 min	42 ± 2 0*** (4)
–	120	15–20 min	82 ± 5 4 (7)
150	120	20–45 min	88 ± 11 5 (5)
150	120	4 hrs	39 ± 5 9 (2)
–	120	4 hrs	46 ± 2 6 (2)

*** $P < 0\,001$ difference versus brain amphetamine level in mice given amphetamine only, and in mice which died at the same time

Table 6

Effect of pretreatment with L-DOPA methylester on amphetamine toxicity and brain and heart amphetamine concentration in mice aged 13–15 days The number of experiments is given in brackets

Pretreatment with H 19/61 (mg/kg)	Dose of amphetamine (mg/kg)	Time of death after amphetamine	Brain amphetamine (µg/g) mean±S E M	Heart amphetamine (µg/g) mean±S E M
150	100	5–10 min	126±16 5 (5)	97±17 9 (5)
–	100	5–35 min	165±16 2 (4)	133± 9 6 (4)
150	100	105–125 min	126±10 7 (2)	38±13 1 (2)
–	100	70–130 min	150±14 4 (4)	64±13 0 (4)
150	100	4 hrs	55± 8 7 (4)	18± 3 1 (4)
–	100	4 hrs	60± 5 5 (4)	16± 3 1 (4)

In mice aged 13–15 days, 100 mg/kg of amphetamine given to pretreated mice induced a mortality rate of 7/16 In unpretreated mice it was 9/16 From table 6 it can be seen that also in this age group the pretreated mice which died spontaneously after amphetamine showed somewhat lower brain and heart amphetamine concentrations than mice injected with amphetamine only During 4 hours the brain and heart amphetamine was decreased to the same level in both groups

Pretreatment with L DOPA enhanced the toxic effect of 60 mg/kg of amphetamine in adult mice The mortality rate in the pretreated mice was

Table 7

Effect of pretreatment with L DOPA methylester on amphetamine toxicity and brain amphetamine concentration in adult mice The number of experiments is given in brackets

Pretreatment with H 19/61 (mg/kg)	Dose of amphetamine (mg/kg)	Time of death after amphetamine	Brain amphetamine (µg/g) mean±S E M
150	60	30 min	135±5 1 (3)
150	60	80 min	115±6 8 (2)
150	60	145–215 min	36±6 0 (6)
–	60	130–165 min	36±23 8 (2)
150	60	4 hrs	12±3 8 (4)
–	60	4 hrs	[illegible]

12/16 and in the mice injected with amphetamine only, 2/12 The brain amphetamine concentration was the same in animals which died spontaneously in both groups, as well as in survivors decapitated 4 hours after amphetamine (table 7)

In all the experiments performed with both developing and adult mice the dose of L-DOPA methylester induced a marked sedation, which was most pronounced immediately after the injection and almost completely over in 60 minutes The stimulating effect of amphetamine after the pretreatment seemed to be somewhat milder than without any pretreatment

Discussion

The brain CA-changes in response to L-DOPA methylester differ between developing and adult mice as was also found after the administration of methyltyrosine methylester (ALHAVA 1973) As shown in a previous study (ALHAVA 1973), the 13-15-day-old mice seemed to be at a particular stage of development as regards the brain DA levels The DA pool of this age group was particularly resistant to the inhibition of tyrosine hydroxylase (ALHAVA 1973), and after L-DOPA a marked overshooting of the DA level could be seen The decarboxylation of L DOPA appears to be effective in the developing organism The manyfold increase in the DA level in both age groups of developing mice might reflect a relative lack of dopamine β hydroxylase as well as a low activity of both CA-degrading enzymes in the developing brain (MIRKIN 1970) Furthermore, the insufficient development of the granular storage system in young age groups could partially explain this phenomenon The present findings give additional support to the existence of the previously suggested differences in the turnover rates of CA during development (ALHAVA & KLINGE 1972 ALHAVA 1973)

As to the ability to degrade L DOPA methylester, the 13-15 day-old mice again showed their particular type, having only 2 7 % of the drug as methyl ester at both times after the administration This reflects a high hydrolytic activity in this age group and is in agreement with the findings of DONE (1964), who mentioned that the hydrolytic reactions of developing animals occur as actively as those of mature animals In this respect the infant mice lie between adult and 13 15-day old mice, which might indicate a relative immaturity of the general metabolic processes in very young animals (DONE 1964, MIRKIN 1970)

The hypokinesia seen after the administration of H 19/61 in adult and developing mice tallies with the finding of STRÖMBERG (1970), who revealed a dose dependent reduction in motor activity in mice injected with L-DOPA

in a dose range of 31 3 to 250 mg/kg The mechanism of this sedation is probably central, since it can be potentiated by inhibiting extracerebral DOPA decarboxylase (STRÖMBERG 1970), and since the brain DA level and the time course of hypokinesia roughly correlate with each other

Large doses of amphetamine induced very high brain DA levels in developing mice pretreated with L-DOPA Since L-DOPA alone was far less active in this respect, the amphetamine effect might result from the dopamine β hydroxylase-inhibiting activity of amphetamine (GLOWINSKI *et al* 1966) Considering the high doses of amphetamine used, i e producing high levels of amphetamine in the brain, the possibility of MAO inhibition cannot be excluded (GLOWINSKI *et al* 1966, RUTLEDGE 1970) This seems even more likely since the activity of the CA-inactivating enzymes in developing animals is low (MIRKIN 1970) As previously discussed (ALHAVA 1973), one further reason for increased DA levels might be the stimulation of biosynthesis of brain DA induced by amphetamine (COSTA & GROPPETTI 1970)

In pretreated adult mice, the CA changes of which in response to L-DOPA alone were much milder than those of developing animals, a considerable depletion of both amines was seen at 4 hours after amphetamine injection This might be explained by the different turnover rate of CA in the mature brain Furthermore, the short duration of the peak concentration of amphetamine in adult brain in contrast to the infant brain (ALHAVA & MATTILA 1972) may exclude the inhibition of MAO activity by amphetamine at the very time of the brain CA assay The high level of DA in pretreated adult mice killed by 100 mg/kg of amphetamine is assumed to result from either the persistence of L-DOPA effect at the time of death or from the same effects of amphetamine on DA turnover rate as discussed above, or both

In a previous study (ALHAVA 1972) it was suggested that in developing mice the immature adrenergic system may limit the development of hyperthermia and hence diminish the exhaustion component of toxicity The present findings support this assumption, since the typical amphetamine-induced hyperthermia seen in adult mice could not be reproduced in developing mice FUXE & SJOQVIST (1972) have suggested the existence of a critical balance between central DA and NA neurons in the thermo-regulation of the mouse, and hence, a selective direct stimulation of DA receptors would elicit a decrease in body temperature, whereas stimulation of NA receptor activity would tend to increase the body temperature In 13 15 day-old mice the brain ratio for DA/NA was 1 2 while the respective ratio in adults was 2 1 (ALHAVA & KLINGE 1972) Amphetamine in turn affected brain DA more easily than brain NA in young mice Hence, it is tempting to suggest that amphetamine hypothermia in 13-15-day-old mice could be primarily due to DA release, and, secondarily, to the reuptake-blocki

activity of the drug (GLOWINSKI *et al* 1966) the combined effects thus leading to an increased amount of DA at the receptor sites The potentiation of hypothermia by L-DOPA, too, could be explained by an increased availability of DA at the receptor sites The predominance of DA release over that of NA in this age group is further supported by the very greatly increased DA levels after L-DOPA given either alone or together with amphetamine

As previously shown (ALHAVA & KLINGE 1972), in adult mouse brain 50 mg/kg of amphetamine depleted the NA level to 35 % of the control level in 4 hours, whereas DA was reduced only to 90 % Accordingly, the hyperthermia seen after this dose of amphetamine in adult mice might be explained by a primary increase in NA at the receptor sites, which may dominate DA release in the brain (FUXE & SJOQVIST 1972) The short duration of initial hypothermia seen after the administration of L-DOPA correlates with the time course of brain DA level after H 19/61, both effects reaching their maximum at 30 minutes after the injection (table 1, fig 3) Thereafter a balance between DA and NA neurons in thermoregulation is reached (FUXE & SJOQVIST 1972) Perhaps due to the short duration of the L DOPA effect on the DA level, the increase in body temperature after amphetamine in spite of pretreatment with H 19/61 is again observed In infant mice, the general immaturity of the adrenergic system as well as the lack of fur might explain the insufficiency of thermoregulation Further discussion about the temperature control of mice is not justified because of the relative lability of thermoregulation of this animal species

As reported by several investigators (RANDRUP & MUNKVAD 1966, HANSON 1967), L-DOPA and amphetamine are known to potentiate each other, and in reserpinized mice the amphetamine toxicity is markedly increased after pretreatment with L-DOPA (MENON & DANDIYA 1967) With regard to adult mice, the present findings support this finding, and the enhancement of amphetamine toxicity by L DOPA was also reproduced in infant mice Even though the pretreatment did not considerably change the mortality rates of developing mice as compared with those induced by amphetamine only, the H 19/61 pretreated infant mice after death showed a lower brain amphetamine concentration The difference between tissue concentrations within the two groups of treatment is so remarkable that it cannot be explained by the slight interference with amphetamine recovery caused by H 19/61 The slow development of the peak of brain amphetamine concentration in infant mice is analogous with the previous finding from this laboratory (ALHAVA & MATTILA 1972) It appears that the initial massive overload of DA in the brain could change the brain perfusion and thus inhibit the penetration of amphetamine into the brain tissue Hence, in the presence of a DA overload, even small concentrations of amphetamine in

the brain could be sufficient to induce death, e g by causing an acute massive release of CA (CARLSSON 1970) The short duration of the L DOPA effect might explain the disappearance of the difference in tissue concentrations with time

Pretreated 13-15-day-old mice killed by amphetamine showed a tendency to lower tissue amphetamine concentration than those injected with amphetamine only, although the difference was insignificant The insignificance is considered to result from the great variability in the time at which death occurred between the two groups of treatment Therefore, it is probable that the same phenomenon as in infant mice in amphetamine toxicity is also involved in L-DOPA pretreated mice of this age In adult mice, in which the pattern of brain amphetamine concentration is known to be different (ALHAVA & MATTILA 1972), even the mortality rates showed the potentiating effect of L-DOPA on amphetamine toxicity Furthermore, since the effect of L-DOPA on brain DA level is much milder than in developing mice there is no such hindrance induced by a DA overload with regard to the penetration of amphetamine into the brain and a higher concentration of the drug is needed to induce early death In the mice which died 2 to 3 hours after the treatment, who already showed a considerably reduced brain amphetamine level, further changes in the central CA turnover rate possibly lead to a lethal outcome

The present findings again reveal the complexity of the amphetamine action They also give further support to the assumption previously made (ALHAVA 1973) about the particular stage of biochemical development of the 13-15 day-old mouse brain Furthermore, the effects of L DOPA may be quite variable depending on the dosage (HORNYKIEWICS 1966, STROMBERG 1970), which complicates the interpretation of the L-DOPA data It appears that the toxic action of amphetamine in developing organism might be based on changes in those enzyme levels which are responsible for the CA turnover rate Further conclusions from the present results may not be justifiable without more specialized biochemical experiments

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A Comparative Study on the Effect of Fluoride, Laurylsulphate and Chlorhexidine on Glucose Utilization in Rat Intestinal Mucosal Cells

By

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Abstract Suspensions of rat intestinal cells were incubated for 30 min at 38° with either sodium fluoride, sodium laurylsulphate or chlorhexidine acetate Except for a slight stimulation with fluoride and laurylsulphate at the lowest concentration tested (0 05 mM), all three compounds progressively suppressed glucose utilization at increasing concentrations A 50 % inhibition occurred at approximately 3 mM fluoride, 1 mM laurylsulphate and 0 2 mM chlorhexidine Thus on an equimolar basis fluoride proved to be the least potent inhibitor of mucosal glucose metabolism The implications of these findings are discussed with regard to the concentrations of these compounds used in oral therapeutics as well as to the amounts that may be swallowed and thereby reach the intestinal epithelium According to the results from this *in vitro* system, the amounts of fluoride tested are less likely to affect this essential digestive process than the corresponding quantities of laurylsulphate or chlorhexidine

Key words Fluoride – laurylsulphate – chlorhexidine – glucose metabolism – rats – intestinal cells

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The use of fluoride represents a well-established aid in providing more decay-resistant teeth The amounts and concentrations in current use depend on the mode of administration or application In dentifrices the concentration of the fluorine component usually corresponds to approximately 0 1 % fluoride

Synthetic anionic detergents such as laurylsulphate in concentrations of 1–3 % are also common dentifrice components but have a less well-proven value

Recently it has been demonstrated that local application of chlorhexidine in the oral cavity exerts a remarkable dental plaque-preventing effect (Löe & Schiøtt 1970) Dentifrices containing 0 6 % and 0 8 % chlorhexidine have been tested in clinical trials (Gjermo & Rölla 1970)

Drugs used in dentifrices or mouth rinses may not have only the intended local effect, as considerable amounts may reach the gastrointestinal tract After a two minute mouth rinse with 7 ml of 0 05 % sodium fluoride solution, 16±4 % of the fluoride was retained in the body (BIRKELAND & LÖKKEN 1972) The retention of chlorhexidine after one-minute rinses with 10 ml of 0 05 % chlorhexidine digluconate solution amounted to 43±7 % (BONESVOLL *et al* 1972), and with 10 ml 0 2 % sodium laurylsulphate solution to 27±6 % (BONESVOLL *et al*, personal communication 1972)

Next to the oral epithelium, the gastrointestinal mucosa will be exposed to the highest concentrations of ingested substances The present investigation gives an *in vitro* comparison of the potency of fluoride, laurylsulphate and chlorhexidine with respect to their interference with one of the essential digestive processes, namely the glucose metabolism of the intestinal epithelium

Material and methods

Non fasting male rats (180–220 g) were anaesthetized with urethan intraperitoneally using 1 0 ml of an aqueous 12 5 % urethan solution per hundred gram body weight The intestinal mucosal cells were then isolated as described by SOGNEN (1967) and incubated in a Krebs Ringer phosphate solution

To 25 ml Erlenmeyer flasks containing 100 µl (0 05 µci) D glucose $^{14}C(U)$ (The Radiochemical Centre Amersham) was added sodium fluoride sodium laurylsulphate or chlorhexidine acetate in a 100 µl volume or in the controls 100 µl 0 9 % NaCl Of the cell suspension 2 8 ml was then pipetted into each flask Thereafter a centre glass well with 200 µl Hyamine® (Packard Instrument Co) for the collection of $^{14}CO_2$ was put into the flasks which were then oxygenated for 4 min The flasks were sealed with rubber caps (Capuchons Alpha Pour Sterilisateur) and left in a shaking bath for 30 min at 38° Incubation was terminated by adding 0 5 ml 75% trichloroacetic acid to stop the metabolism and to release carbon dioxide from the solution Shaking continued for 5 min. The vessels containing Hyamine were then transferred *in toto* to counting vials with 10 ml Insta Gel® (Packard Instrument Co) and counted in a Packard Tri Carb Scintillation Spectrometer model 3310

Results

The effect of fluoride laurylsulphate and chlorhexidine on glucose metabolism in isolated intestinal mucosal cells is illustrated in fig 1 Chlorhexidine proved to be the most potent inhibitor and caused an almost complete suppression at 0 5 mM, while with laurylsulphate a concentration about ten times higher was needed to obtain this effect Fluoride was still less potent At 0 05 mM concentrations of fluoride and laurylsulphate, glucose utilization appeared to be slightly stimulated This tendency was not observed with any of the tested concentrations of chlorhexidine

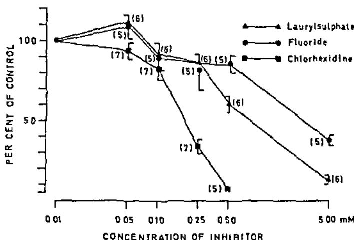


Fig 1 The effect of fluoride laurylsulphate and chlorhexidine on glucose utilization in rat intestinal mucosal cells During incubation for 30 min at 38°, $^{14}CO_2$ formed from metabolized D glucose ^{14}C was collected The values (mean values ± S D) are calculated as percentages of the controls (In brackets, number of experiments)

sulphate at low concentrations has previously been reported to stimulate rat intestinal glucose absorption (NISSIM 1962) and respiratory rates in suspensions of rat hepatic cells (TARNOFF & STRAUSSER 1969) DAVIES (1949) found that the respiration of frog gastric mucosa was elevated by low levels of fluoride

Discussion

The question whether daily ingestion of small quantities of substances, such as those presently tested, interferes with gastrointestinal metabolism and absorption of nutrients or drugs is complex Dilution and binding to proteins and other components in the alimentary fluids may reduce or modify their effects The very rapid regeneration of the intestinal mucosa may further contribute to a reduction of the effect on this epithelium Usually, the mucosal transport capacity for nutrients exceeds by far the body requirements, and it is not likely that a minor deficiency in the uptake will be of any toxicological importance under normal conditions In alimentary diseases or at exceptionally high requirements, however, the presence of interfering substances may become a problem

Mucosal glucose metabolism, selected as our test parameter, represents an essential energy-yielding process for a number of active transport mechanisms, e g of fluid (BARRY *et al* 1961), sodium (BARRY *et al* 1967), amino acids (DAWSON *et al* 1965), calcium (SCHACHTER & ROSEN 1959), and of

glucose itself Inhibition of glycolysis may therefore be regarded as an expression of a general toxic effect on the intestinal mucosal cells

Fluoride ions are generally considered to be a potent inhibitor of glucose metabolism In the *in vitro* system used at present chlorhexidine proved to be a more potent inhibitor of this metabolic process than either fluoride or laurylsulphate A 50 % inhibition of glucose metabolism occurred at approximately 0 2 mM (0 0125 %) chlorhexidine, 1 mM (0 029 %) laurylsulphate, and 3 mM (0 0126 %) fluoride

The concentration of chlorhexidine, which in the present study inhibited glucose metabolism, corresponds to the results of HELGELAND *et al* (1971), who found toxic effects of chlorhexidine in concentrations from 0 05 mM in cultures of human epithelial cells

The results of this comparative study may be of interest when related to the concentrations and amounts of the drugs that are used in dentifrices and mouth rinses While the possible deleterious effects of the caries prophylactic use of fluoride have received much attention, less interest has been focused on the potential of other agents of more questionable value, such as laurylsulphate The present results indicate that, compared to both laurylsulphate and chlorhexidine, the amount of fluoride that may be ingested following the use of such products is less likely to interfere with glycolysis in intestinal mucosal cells It should be stressed, however, that care must be taken when interpreting these *in vitro* findings, as differences in their affinities for inactivating components *in vivo* may modify the relative toxicities of the agents tested

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The Comparative Pharmacology of Flupenthixol and some Reference Neuroleptics

By

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Abstract Flupenthixol a thioxanthene derivative may exist in two isomeric forms α and β Flupenthixol contains 45-55 % α flupenthixol The pharmacological effects of flupenthixol α and β flupenthixol have been compared with those of clopenthixol chlorprothixene fluphenazine perphenazine, chlorpromazine and haloperidol In most pharmacological screening tests α flupenthixol was equipotent with fluphenazine β Flupenthixol showed very low pharmacological activity As expected the potency of flupenthixol was about one half that of α flupenthixol Flupenthixol was considerably more potent than clopenthixol and chlorprothixene in the inhibition of conditioned avoidance response The influence on temperature regulation and the peripheral α adrenolytic effect was considerably weaker than that of chlorprothixene

Key words Pharmacology – psychopharmacology – tranquilizing agents – flupenthixol – clopenthixol – chlorprothixene

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In our original publication (Møller Nielsen *et al* 1962) on the central depressant activity of thioxanthenes, there was no evidence with the methods used in this study, that flupenthixol (N 7009) was more active than many other substances Since then, as other more specific methods have been tried, flupenthixol appeared to be a potent neuroleptic, and the most potent in the thioxanthene series Clinically, flupenthixol has been established as a potent, low dose neuroleptic It has a very low sedative-hypnogenic potency, while on the contrary, a certain activating effect has been ascribed to this drug In the present paper the psychopharmacological profile of flupenthixol has been compared with that of ι ' chlorprothixene, clopenthixol, perphenazine, fluphenazine and halc (formulas see fig 1)

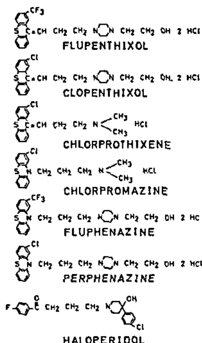


Fig 1 Formulas of flupenthixol and reference substances

Like other ring-substituted thioxanthenes, flupenthixol may exist in two isomeric forms, α and β Flupenthixol contains 45–55 % α flupenthixol For this pharmacological study α- and β flupenthixol were prepared in a pure form and tested separately

Materials and methods

Compounds

Flupenthixol α flupenthixol β-flupenthixol clopenthixol perphenazine and fluphenazine were available as the dihydrochlorides chlorprothixene and chlorpromazine as hydrochlorides These substances were used as aqueous solutions Haloperidol was dissolved in 0 1 M tartaric acid and diluted as required with distilled water All doses refer to the respective substances according to weight

Animals

Except where otherwise stated the following animals were used
Male mice, NMRI/BOM
Male rats, Wistar/Af/Han/Mol (Han 67)
Guinea pigs unspecified either sex
Rabbits New Zealand White either sex
Cats unspecified domestic cat either sex
Dogs pure bred Beagles, own breed either sex

Calculations

Most log probit analyses and calculations of 95 % confidence limits were done by means of an IBM 1130 computer system according to the method described by FINNEY (1952)

Methods

Inhibition of spontaneous motor activity in mice

Motility was determined in jiggle cages measuring the amount of exploratory activity of mice placed in a new environment for 15 minutes The method used was essentially the same as that described by KOPF & MOLLER NIELSEN (1959) The jiggle cages have been modified so that vibrations are registered by means of a strain gauge bridge ED50 was defined as the dose reducing the activity to 50 % of that of the control animals Ten animals were used per dose level and 20 animals for the control groups Test substances were administered intraperitoneally

Cataleptic reaction, vertical rod test in mice

Mice weighing 25–28 g were kept in groups of five in Macrolon cages, type 2 at a room temperature of 23°

A modification of the method described by ZETLER *et al* (1960) was used to measure the cataleptic reaction In this test the animals were considered cataleptic when they remained immobile during a period of 30 sec after being placed on a vertical rod wound with string Untreated mice climbed normally up and down the rod Mice treated with cataleptogenic drugs at appropriate dose levels remained immobile on the rod The animals were checked for the presence or absence of catalepsy ½, 1, 1½, 2, 2½, 3, 4, 5 and 6 hrs after subcutaneous injection of the test compounds Five animals were used at each dose level ED50 values, defined as the doses producing catalepsy in 50 % of the mice, were determined on the basis of the maximal cataleptic effect of each dose level, irrespective of time

Cataleptic reaction (wire mesh) in rats

Rats were kept in groups of 5 in Macrolon cages, type 3, at a room temperature of 22.5 to 23 5°

The animals were placed on a vertical wire mesh Rats were considered cataleptic when they remained immobile during a period of 15 seconds Untreated rats climbed normally up and down the wire mesh

The animals were checked for the presence or absence of catalepsy 1, 2, 3, 4, 5 and 6 hrs after intraperitoneal injection of the test compound The ED50, defined as the dose producing cataleptic reaction in 50 % of the rats, was calculated for each time of observation The $ED50_{min}$ was defined as the lowest ED50 that could be obtained in relation to time during 6 hours of testing

Ptosis inducing effect in mice

In order to differentiate between spontaneous and drug induced ptosis a light exteroceptive stimulation was applied (TEDESCHI 1967) A Macrolon cage, type 2 containing 5 mice was tilted up and down once Thirty seconds later the degree of ptosis was determined according to the scoring system adopted by RUBIN *et al* (1957) Each group of mice was tested ½, 1, 1½, 2, 2½, 3, 4 and 5 hrs after intraperitoneal inject of the test compound

The ptotic score for each dose group and time interval was expressed in per cent of the maximal obtainable score The ED50 was determined on the basis of maximal per cent ptosis for each dose level, irrespective of time

Antagonism against apomorphine induced stereotypy in rats

Rats (230–270 g) were given test substance intraperitoneally (5 animals per group) Two hours later apomorphine hydrochloride (10 mg/kg) was injected intravenously and the animals were placed in individual cages for one hour A cage consisted of a 30 cm high perspex box (12 × 25 cm), without bottom and lid, which during the experiment was placed on corrugated paper In this situation control animals, when given apomorphine, exhibit excessive gnawing compulsion In the experimental groups the percentage of animals that did not bite the corrugated paper was determined ED50 values, defined as the dose protecting 50 per cent of the animals against gnawing compulsion were calculated

Antagonism against apomorphine induced vomiting in dogs

The threshold dose of apomorphine hydrochloride for the induction of vomiting in our dogs has been determined to be 25 μg/kg intravenously After this dose vomiting occurs within a few minutes

Test substances were given at 3 to 4 dose levels subcutaneously (2 to 4 dogs per dose level) Four hours later the dogs were challenged with the threshold dose of apomorphine hydrochloride and observed for the occurrence of vomiting. Approximate ED50 values were determined

Antagonism against apomorphine induced stereotypy in dogs

When given 1 6 mg/kg apomorphine hydrochloride intravenously, dogs display a stereotyped running activity which starts a few minutes after the injection and persists for about 1 hour (NYMARK 1972) Test substances were injected subcutaneously in 2 to 6 animals per dose level 4 hrs before the apomorphine challenge The animals were then observed for the presence or absence of stereotyped running activity for 1 hour Approximate ED50 values for protection were determined

Antagonism against amphetamine induced stereotypy in rats

Rats (230–270 g) were given a subcutaneous dose of the drug under investigation and immediately thereafter 13 6 mg/kg of amphetamine sulphate (~10 mg/kg of amphetamine base) was injected intravenously The animals were then placed in individual cages as described above under apomorphine antagonism After 55 and 65 minutes the rats were observed for stereotyped movements of head and forelegs for one minute The absence of stereotypy was interpreted as a drug effect ED50 values for protection against stereotypy were determined

All compounds were tested at a minimum of three dose levels using 10 rats per dose and the observer was kept unaware of the identity of the groups until after the experiment

Antagonism against methylphenidate induced stereotypy in mice

Mice (18–25 g) were given an intraperitoneal dose of the test compound Two hours later 60 mg/kg of methylphenidate hydrochloride was injected subcutaneously, and immediately thereafter the animals were placed in cages, 2 mice in each cage, for 1 hour The cages which during the experiment were placed on corrugated paper, are described above under apomorphine antagonism In this situation control mice when

given methylphenidate, bite the corrugated paper (gnawing compulsion) (PEDERSEN & CHRISTENSEN 1971 & 1972) All compounds were tested at at least 3 dose levels using 3 to 6 pairs of mice per dose The absence of biting the paper was considered as a drug effect ED50 values were calculated

Inhibition of conditioned avoidance response, rats

Rats were trained to avoid an electrical shock in a shuttle box (Ugo Basile, Milan) by moving from one compartment to the other during a period of warning Each trial was organized as follows 5 sec. of warning signal (tone), 5 sec of tone + shock and 20 sec intertrial An experimental session consisted of 10 such trials Change of side immediately would terminate the stimulus being presented and thus prolonging the intertrial period All experiments were controlled by a Massay Dickinson programming and recording system

During training and testing three types of responses were scored if the rat crossed in response to the conditioned stimulus (tone) only, a "conditioned avoidance response" (CAR) was scored, if the cross occurred during the time when both the conditioned and the unconditioned stimuli (tone and shock) were being presented, an "unconditioned escape response' (UER) was scored, if the rat failed to cross during the shock period, an 'escape failure" (EF) was scored

All rats were trained until a criterion of at least 90 % avoidance response was reached Five or ten rats were used at each dose level For each substance tested a new group of rats were trained The rats were tested 1 hr before and 1, 2, 3, 4, 5, 6 and 24 hrs after subcutaneous administration of the test substance

The per cent inhibition of CAR was calculated on the basis of the pre-dosing level The per cent inhibition of UER was calculated as follows $\frac{EF}{N\ 10}$ 100 (EF = total number of escape failure, N = number of animals tested) ED50 values were cal culated

Influence on body temperature, rats

The body temperature of individually caged, restrained rats was recorded by means of a thermocouple (El lab, Copenhagen) The room temperature was 22–24° The temperature was measured 45 minutes before and ¼, ½, 1, 1½, 2 3, 4 and 5 hrs after intraperitoneal injection of the test compound at dose levels of 1 25, 2 5 and 5 mg/kg

Adrenaline (A) noradrenaline (NA) and 5 hydroxytryptamine (5 HT)-antagonism pithed rat preparation

The antagonism to A, NA and 5 HT pressor effects in pithed rats was studied by the method previously described (MØLLER NIELSEN & NEUHOLD 1959) Test compounds were injected intravenously at 3 to 4 dose levels (n = 2 to 4) Reduction of pressor response was calculated in per cent of the initial value The per cent inhibition was plotted against log dose, and the ED50 read on the diagram In the case of NA antagonism ED50 could not always be determined, but instead an ED35 was obtained

α Receptor blocking effect denervated nictitating membrane, anaesthetized cat

In 10 cats the right nictitating membrane was denervated by removal of
cervical ganglion under mebumal Na (pentobarbital Na) anaesthesia. T
later the cats were anaesthetized with chloralose urethane (43 and 430

peritoneally + ⅔ intramuscularly) The contractions of the denervated nictitating membrane was recorded by means of a Sanborn displacement transducer (Type 7DCDT 1000) Load 6 5 g and magnification 10 times Recordings were made on an ultraviolet galvanometer writer (ABEM Ultralette 12) The right femoral vein was canulated for injection of the drugs A standard dose of noradrenaline bitartrate corresponding to 5 µg/kg base was injected intravenously every 15 minutes Increasing doses of the test compound were given intravenously at 45 minute intervals Inhibition of the NA response was calculated in per cent of the initial value Dose effect curves were plotted on semilogarithmic paper The ED50 values corresponding to 50 % inhibition were read on the diagrams

Anticholinergic effect guinea pig ileum preparation

The influence on the acetylcholine evoked contractions of the isolated guinea pig ileum was determined according to the method of MAGNUS (1904)

In each experiment varying doses of the test substance and of atropine sulphate were given in random order The antagonistic effect of the test substance was calculated in relation to that of atropine sulphate Acetylcholine HCl was added to the bath in a final concentration of 0 04 µg/ml

Results and discussion

The results obtained with flupenthixol, α- and β flupenthixol and 6 reference neuroleptics, in 13 screening tests are listed in table 1 It can be seen that α-flupenthixol in most respects compares well with fluphenazine With regard to ptotic activity it is about 3 times more potent The antagonism against apomorphine stereotypy in the dog is only one third of that of fluphenazine while in rats the apomorphine antagonism of the two compounds is about equal Both are equally potent antiemetics in dogs The adrenaline antagonism of α-flupenthixol was considerably stronger than that of fluphenazine, though it was not possible to demonstrate a noradrenaline antagonistic effect with α-flupenthixol Surprisingly a noradrenolytic effect could be determined with flupenthixol although α- and β flupenthixol were almost without activity The anticholinergic effect of α- and β flupenthixol was very low and of the same order as that of clopenthixol, fluphenazine, perphenazine and haloperidol, chlorprothixene being the most potent anticholinergic drug

The inhibitory effect of flupenthixol, clopenthixol and chlorprothixene on conditioned avoidance response (CAR) and unconditioned escape response (UER) is illustrated in fig 2 It can be seen that all three neuroleptics inhibited CAR at doses lower than those causing escape disruption Flupenthixol was the most active, the lowest ED50 being 0 06 mg/kg subcutaneously Surprisingly clopenthixol was only slightly more active than chlorprothixene, the lowest ED50 values being 0 24 and 0 33 mg/kg subcutaneously, respectively Peak effect of flupenthixol occurred at 4 hrs The 24 hrs ED50 value was 3 times that of the lowest ED50

Table 1.

Test	Motility	Catalepsy rod	Catalepsy WM	Ptosis	Apomorph stereo	Apomorph stereo	Apomorph vomit	Amphet. stereo	Methylph stereo	Noradr antagon	Adren antagon.	5 HT antagon	Ach antagon
Species*	M	M	R	M	R	D	D	R	M	R	R	R	GP
	ED50 mg/kg i p	ED50 mg/kg i p	ED50 mg/kg i p	ED50 mg/kg i p	ED50 mg/kg i p	ED50 mg/kg s c	ED50 mg/kg s c	ED50 mg/kg s c	ED50 mg/kg i p	ED35 mg/kg i v	ED50 mg/kg i v	ED50 mg/kg i v	A=100**
Flupenthixol	1.3	0.5	0.3	0.9	0.5	0.08	0.02	0.2	0.15	0.4	0.17	0.06	0.06
α Flupenthixol	0.6	0.3	0.1	0.3	0.3	0.06	0.01	0.07	0.07	–***	0.02	0.02	0.05
β Flupenthixol	22	16	77	33	>80	–	3.5	>160	2.5	–***	4.8	2.8	0.1
Clopenthixol	1.1	0.7	0.9	1.3	30	3.2	0.04	0.2	1.2	0.04	0.03	0.03	0.07
Chlorprothixene	0.4	1.8	2.3	1.6	45	6.0	0.9	0.5	0.7	0.03	0.01	0.004	3.3
Fluphenazine	0.8	0.3	0.1	0.9	0.2	0.02	0.01	0.08	0.04	0.3	0.35	0.07	0.1
Perphenazine	0.6	0.4	0.2	0.9	0.5	0.6	0.06	0.09	0.06	1.3	0.14	0.06	0.08
Chlorpromazine	2.1	1.9	5.5	1.3	59	>20	0.6	0.6	6.2	0.15	0.05	0.05	0.5
Haloperidol	0.2	0.7	0.4	0.6	2.6	0.2	0.01	0.02	0.06	–***	0.67	0.86	0.04

* M=mice, R=rats, D=dogs, GP=guinea pigs
** Relative potency, atropine sulphate=100
*** ED35 could not be determined

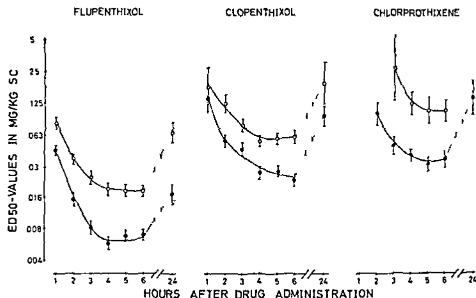


Fig 2 Inhibition of conditioned avoidance response (CAR) and unconditioned escape response (UER) of flupenthixol, clopenthixol and chlorprothixene in rats ●——● CAR, ○——○ UER Vertical bars show 95 % confidence limits

In fig 3 is shown the influence of flupenthixol and four reference substances on body temperature Most potent was chlorprothixene followed by clopenthixol and chlorpromazine, while flupenthixol and fluphenazine showed only moderate temperature reducing effect

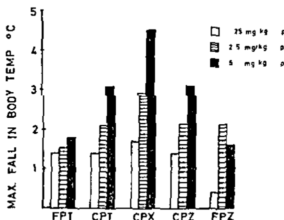


Fig 3 Influence on body temperature of rats FPT = flupenthixol CPT = clopenthixol CPX = chlorprothixene CPZ = chlorpromazine FPZ = fluphenazine

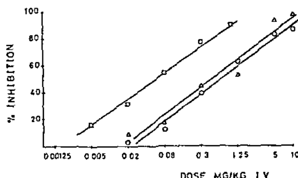


Fig 4 Inhibition of NA response in the denervated nictitating membrane preparation anaesthetized cats ○ Flupenthixol △ clopenthixol □ chlorprothixene

The three thioxanthenes have been compared with regard to α-adrenolytic effect on the denervated nictitating membrane All three substances caused a dose dependent reduction of the contraction of the nictitating membrane on intravenous injection of NA (5 μg/kg) In fig 4 are presented the dose response curves for flupenthixol, clopenthixol and chlorprothixene, the corresponding ED50 values were 0 62, 0 48 and 0 06 mg/kg intravenously, respectively Thus flupenthixol and clopenthixol showed a considerably weaker α adrenolytic effect than did chlorprothixene

This study has confirmed the previous findings with thioxanthenes, that the stereoisomeric configuration is of decisive importance for the pharmacological activity (Møller Nielsen *et al* 1962) α Flupenthixol was found largely equipotent with fluphenazine, while β flupenthixol showed a very low activity Since β flupenthixol has this very low level of activity, the potency of flupenthixol, which contains about 50 % of α flupenthixol, should be expected to be about half that of α flupenthixol This also seems to be the case

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Prolonged Neuroleptic Effect of α-Flupenthixol Decanoate in Oil

By

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Abstract The duration and intensity of the neuroleptic effect of α flupenthixol decanoate in viscoleo® have been compared with that of α flupenthixol, 2 HCl in aqueous solution and α flupenthixol base in viscoleo® in a number of animal experimental models Incorporation of α flupenthixol base in oil led to a prolongation of the neuroleptic effect (apomorphine antagonism in dogs, inhibition of conditioned avoidance in rats and mice) as compared to the duration of the effect of α flupenthixol, 2 HCl in aqueous solution In both cases marked sedation was observed In contrast, there was no sedation after α flupenthixol decanoate in oil but a slower, smoother onset and a much longer duration of neuroleptic effect resulted In rats the cataleptic effect and the apomorphine antagonistic effect of α flupenthixol decanoate in oil were of much shorter duration than the inhibitory effect on conditioned avoidance response In mice a slight potentiation of barbiturate anaesthesia could be demonstrated 1–6 hrs after high doses of α flupenthixol decanoate in oil. The clinical advantages of the depot preparation are discussed

Key words Tranquilizing agents – delayed action preparations – flupenthixol – psychopharmacology

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Flupenthixol dihydrochloride has been established clinically as a potent neuroleptic drug when administered orally However, in order to maintain sufficient drug concentration at the site of action repeated daily dosages are necessary Such dose regimen affords several difficulties in psychiatric practice (ENERHEIM *et al* 1970)

In order to overcome these difficulties a depot preparation, fluanxol depot® with prolonged action after intramuscular administration has been developed Fluanxol depot® contains as the active principle the ester α-flupenthixol decanoate (for formula see fig 1), dissolved in the low-viscosity vegetable oil, viscoleo® (oleum vegetabile tenue) in a concentration

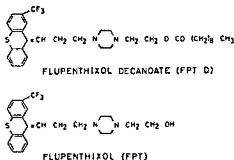


Fig 1 Structural formulas of flupenthixol and flupenthixol decanoate

α flupenthixol decanoate per ml Since α-flupenthixol decanoate is some several thousand times more lipophilic than flupenthixol, 2 HCl (K FREDRICSON OVERØ, unpublished report No 51, Biochemical Laboratory, H Lundbeck & Co A/S, 1970), it is released from the oil depot at a very slow rate and hydrolyzed to α-flupenthixol and decanoic acid in various tissues The metabolic disposition of α-flupenthixol decanoate in oil, injected intramuscularly into rats and dogs has been studied and reported by JØRGENSEN *et al* (1971)

The present paper deals with pharmacological experiments designed to investigate the strength and duration of neuroleptic activity following intramuscular or subcutaneous injections of α-flupenthixol decanoate in oil as compared with that of α flupenthixol, 2 HCl given orally or parenterally and that of α-flupenthixol base in oil administered parenterally

From previous pharmacological experiments (MØLLER NIELSEN *et al* 1973) flupenthixol was known to possess potent neuroleptic activity in a number of animal test models For the present investigation the following models were used Apomorphine antagonism in dogs (antiemetic effect), apomorphine antagonism in rats (antagonism against compulsive gnawing), conditioned avoidance behaviour (shuttle box) in rats and mice, cataleptic reaction in rats and barbiturate potentiation in mice

Being a thioxanthene derivative with an asymmetric ring structure, flupenthixol *may exist in two stereoisomeric forms One isomer, α-flupenthixol* is considerably more active than the β-form (MØLLER NIELSEN *et al* 1973) When preparing the decanoic acid ester from the mixture of α- and β flupenthixol, predominantly the β-isomer is isolated in a crystalline form For this reason the flupenthixol used in the preparation of flupenthixol decanoate was pure α-flupenthixol

Abbreviations used α-flupenthixol dihydrochloride α-FPT, 2 HCl, α flupenthixol base α-FPT, α-flupenthixol decanoate α-FPT-D, α β flupenthixol dihydrochloride FPT, 2 HCl, α-β-flupenthixol base FPT, viscoleo® oil

Materials and methods

Animals

Dogs adult purebred Beagles own breed, either sex
Rats, male Wistar/Af/Han/Mol (Han 67) SPF, conventional housing
Mice, male NMRI/BOM SPF, conventional housing

Apomorphine antagonism in dogs

The threshold-dose of apomorphine hydrochloride for the induction of vomiting in the dogs has been determined to be 25 μg/kg intravenously After this dose vomiting occurs within a few minutes of the injection

Four dogs were used for each dose level of the drug, which was injected subcutaneously at the back of the neck At different times after the drug administration the dogs were then challenged with apomorphine according to an "up and down" schedule using the dose range 25–400 μg/kg intravenously geometrically spaced Thus if for example a dog vomited after 100 μg/kg the next dog was given 50 μg/kg or 200 μg/kg if the first dog did not vomit and so on In this way it was possible to estimate at which level of apomorphine the dogs were protected at a given time The dogs were fed half an hour before testing to ensure an easy vomiting

The following preparations and doses were tested by subcutaneous injection α FPT 2 HCl in aqueous solution (2 %) 2 and 6 mg/kg α FPT in oil (2 %) 2 and 6 mg/kg and α FPT D in oil (2 %) 2 and 6 mg/kg

Apomorphine antagonism in rats

Rats weighing 200-300 g were pretreated with α FPT D in oil (2 %) 8 mg/kg subcutaneously FPT in oil (2 %) 8 mg/kg subcutaneously or FPT, 2 HCl aqueous solution (0 8 %) 8 mg/kg subcutaneously Appropriate control groups receiving injections of the vehicle were included in each experiment In the experiments with FPT in oil and α FPT D in oil 2 groups of 10 rats were used for each preparation The two groups were challenged with apomorphine hydrochloride 10 mg/kg subcutaneously on alternate days the first group 24 hrs and 72 hrs and the second 48 hrs and 96 hrs after the injection In the experiments with FPT, 2 HCl in aqueous solution 3 groups of 10 rats were used These groups were challenged with apomorphine 6 17 or 24 hrs after administration respectively

The testing for apomorphine antagonism was performed as described by Møller Nielsen *et al* (1973) Absence of biting in 30 % or more of the animals was considered a drug effect

Inhibition of conditioned avoidance response

Testing for inhibition of conditioned avoidance response (CAR) and unconditioned escape response (UER) was performed as described by Møller Nielsen *et al* (1973) The following drugs and doses were tested in rats α FPT, 2 HCl in aqueous solution 2 5 and 5 mg/kg (2 5 ml/kg) given orally once daily for 5 consecutive days, α FPT in oil single intramuscular injection of 3 75 and 7.5 mg/kg (0 25 ml/kg) α FPT D in oil single intramuscular injection of 2 5 5 10 and 20 mg/kg (0 25 ml/kg) in mice α FPT 2 HCl (0 5 ml/kg) aqueous solution 0 06 mg/kg single injection intramuscularly the injection being repeated after 48 hrs, α FPT D in oil single intramuscular injection of 5 and 10 mg/kg (0 5 ml/kg)

Cataleptic reaction (wire mesh) in rats

Cataleptic reaction in rats was tested on a vertical wire mesh as described by Møller

NIELSEN et al (1973) Ten rats were used for each dose level of the drugs, which were injected intramuscularly (injection volume 0 25 ml/kg) At different times after drug administration the rats were checked for the presence or absence of catalepsy

Potentiation of enhexymal Na anaesthesia in mice

Mice weighing 18–25 g were used The highest dose of enhexymal Na, which would not induce anaesthesia as defined by loss of righting reflex for more than 1 min, was established to be 32.5 mg/kg intravenously in non pretreated control mice Out of 200 control mice sleep was only induced in 1 animal following this dose of the anaesthetic

Test substance was injected subcutaneously in increasing doses at varying intervals before testing with enhexymal Na Ten animals were used for each dose and time interval Following the injection of the anaesthetic the number of sleeping mice was recorded and expressed as per cent of animals tested

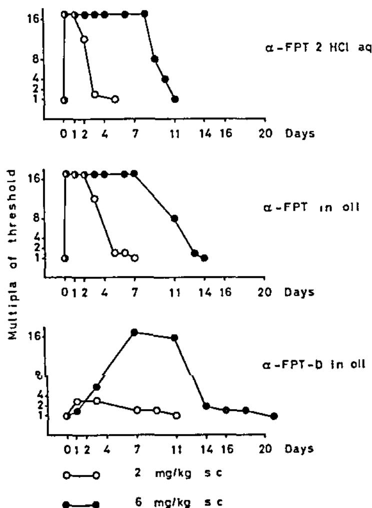


Fig 2 Apomorphine antagonism in dogs

Results

Apomorphine antagonism in dogs

The various preparations of α-FPT all provided pronounced protection against the emetic effect of apomorphine Fig 2 illustrates the time course of the apomorphine protecting effect of the various preparations of α-FPT The ordinate indicates multiples of the normal emetic threshold dose of apomorphine (25 μg/kg intravenously) necessary to cause vomiting at a given time

It appeared that α FPT, 2 HCl 2 mg/kg protected against more than 16 times the threshold dose 1 and 24 hrs after injection Moreover 48 hrs after, a considerable protection persisted Normal threshold was reached between 72 and 96 hrs p i Six mg/kg exerted considerable protection for about 9 days Similarly α FPT in oil, 2 and 6 mg/kg showed very marked protection from 2 hrs to about 4 and 10 days, respectively α FPT-D in oil, 2 mg/kg yielded a more moderate protection, 2–3 times the threshold dose, with maximum value at 24 hrs and reaching normal threshold at about 10 days. Six mg/kg of α FPT-D showed slight protection at 24 hrs The protection increased slowly, reaching maximal value, 16 times the normal threshold, at 7 days p i after which it gradually decreased, reaching normal threshold between day 18 and 21 The animals receiving α FPT, 2 HCl in aqueous solution showed very heavy sedation lasting for 2–3 days Likewise those on α-FTP in oil showed heavy initial sedation gradually subsiding over the following

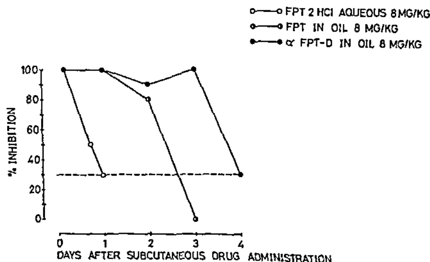


Fig 3 Apomorphine antagonism in rats

2–3 days In sharp contrast, the animals receiving even 6 mg/kg α-FPT-D in oil displayed a completely normal behaviour at all times

Apomorphine antagonism in rats

The results are depicted graphically in fig 3 in which time is plotted against per cent animals protected Although with the method used the effect of all preparations lasted considerably less in the rat than in the dog, a prolongation of effect was demonstrated with FPT in oil and even more so with α-FPT-D in oil as compared with FPT, 2 HCl in aqueous solution The maximal protective effect was seen 4 hrs after the administration of FPT, 2 HCl aqueous and FPT in oil, the effect lasting for about 24 and 60 hrs, respectively α FPT-D in oil was not tested at 4 hrs, but a maximal effect was observed at 24, 48 and 72 hrs A certain degree of protection was still detectable at 96 hrs

It should be noted that the animals receiving FPT, 2 HCl in aqueous solution and FPT in oil were markedly sedated during the first 24 hours, while the animals receiving α-FPT-D in oil exhibited a completely normal behaviour throughout the experimental period

Inhibition of conditioned avoidance response

Rats When tested on drug-free days the rats showed almost 100 % CAR and UER (fig 4) When given α FPT, 2 HCl aqueous solution orally at daily

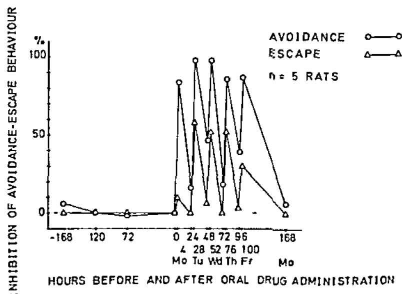


Fig. 4 Inhibition of conditioned avoidance response in rats α FPT, 2 HCl 5 mg/kg perorally daily for 5 days

Table 1

Duration of inhibition of conditioned avoidance response in rats after administration of various preparations of α flupenthixol

Preparation	Dose (mg/kg)	Route	Duration days
α FPT, 2 HCl aqueous	2.5	p o	1–2
	5 0		1–2
α FPT in oil	3 75	i m	7–8
	7.5		11–12
α FPT D in oil	2 5	i m	7–8
	5 0		15–16
	10		22 23
	20		29

doses of 5 mg/kg for 5 consecutive days a marked inhibition (80–100 %) of CAR was registered 4 hrs after each dose (fig 4) Immediately before the next dose the inhibition was considerably lower although an indication of accumulation of effect was seen on days 3 and 5 The UER was inhibited to a lesser degree but also here a certain accumulation of effect was indicated When 2 5 mg/kg was given in the same manner CAR was also considerably inhibited with peak effects of about 50–75 % inhibition

Table 1 gives a survey of the duration of action of the various forms of administration of α FPT While the effect of α-FPT, 2 HCl orally was detectable at most for only 48 hrs, incorporation of α FPT in oil prolonged the effect to 8 to 12 days depending on the dose The α FPT-D in oil exerted

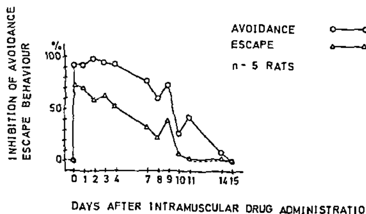


Fig 5 Inhibition of conditioned avoidance response in rats α FPT in oil 7 5 mg/kg intramuscularly

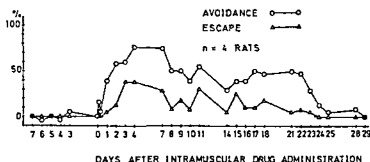


Fig 6 Inhibition of conditioned avoidance response in rats α FPT D in oil 10 mg/kg intramuscularly

an even more marked prolongation of the duration of effect The effects of 5 and 10 mg/kg which are equimolar to 3 75 and 7 5 mg/kg of α FPT lasted for 16 and 23 days, respectively

As examples of the results obtained fig 5 shows the effect of 7 5 mg/kg α FPT in oil and fig 6 that of 10 mg/kg α FPT-D in oil While the effect of α FPT in oil reached its maximum within the first day, that of α-FPT-D in oil gradually increased over the first 4 days and declined at a slower rate With 20 mg/kg FPT-D in oil, however, the maximal effect (100 % inhibition) was reached after 24 hrs and between 100 and 80 % inhibition was maintained for 21 days

The inhibition of UER was relatively high at the beginning after 7 5 mg/kg α FPT in oil and at day 3–4 after 10 mg/kg α-FPT-D in oil

Mice In this species α FPT, 2 HCl in aqueous solution and α-FPT-D in oil were both tested after intramuscular injection The overall results with respect to duration of effect are listed in table 2 With the intramuscular

Table 2

Duration of inhibition of conditioned avoidance response in mice after administration of various preparations of α flupenthixol

Preparation	Dose (mg kg)	Route	Duration days
α FPT, 2 HCl aqueous	0 06	i m	1–2
α FPT D in oil	5 0 10	i m	5–7 11–14

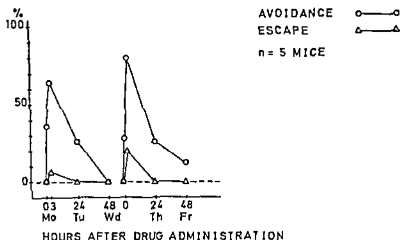


Fig 7 Inhibition of conditioned avoidance response in mice α FPT, 2 HCl, 0 06 mg/kg intramuscularly given twice at 48 hrs interval

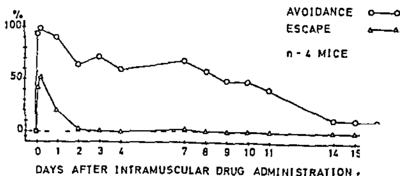


Fig 8 Inhibition of conditioned avoidance response in mice α

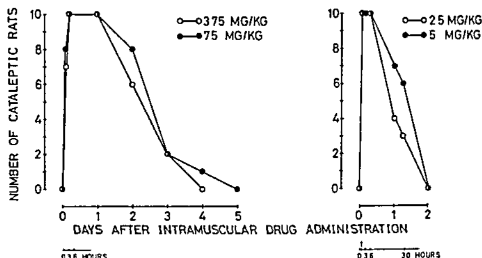


Fig 9 Catalepsy in rats α FPT, 2 HCl aqueous solution and α FPT in oil

route α-FPT, 2 HCl was rather potent since 0 06 mg/kg given twice within the same week yielded a maximum of 60–80 % inhibition of CAR 3 hrs p i (fig 7), the effect being detectable for 24–48 hrs α-FPT-D in oil, 5 mg/kg caused an inhibition, reaching a maximum at about 6 hrs p i and was detectable for 5–7 days With 10 mg/kg α-FPT-D in oil the inhibition of CAR also reached a maximum (100 %) within the first 6 hrs, the effect being detectable for 11–14 days (fig 8) It should be noted that the specificity is rather good in this species since UER was only slightly inhibited by α-FPT, 2 HCl and was very shortlasting (less than 48 hrs) after 10 mg/kg α-FPT-D in oil

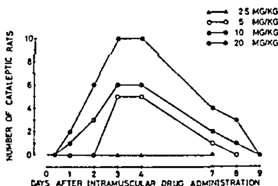


Fig 10 Catalepsy in rats α FPT D in oil

Cataleptic reaction (wire mesh) in rats

The time course of the cataleptic effect of the various preparations of α FPT is illustrated in fig 9 and 10 It appears that α-FPT, 2 HCl, 2 5 and 5 mg/kg, caused 100 % catalepsy 1, 3 and 6 hours p i After 24 and 30 hours some of the animals were still cataleptic, but after 48 hours the effect had completely disappeared α-FPT in oil, 3 75 and 7 5 mg/kg also caused 100 % catalepsy and a maximal effect was reached within 6 hours The duration of action was 3–5 days

α-FPT D in oil, 2 5 mg/kg did not induce catalepsy With the doses 5 and 10 mg/kg, which are equimolar with 3 75 and 7 5 mg/kg of α-FPT, a cataleptic reaction could be detected, the maximal effect being 50 and 60 % depending on the dose Only the high dose, 20 mg/kg, caused 100 % catalepsy The maximal effect of α-FPT-D in oil was reached within 3 days The duration of effect was 7 to 8 days

The animals treated with α-FPT, 2 HCl and α-FPT in oil were heavily sedated during the first 24 hours After α-FPT-D in oil only the animals treated with the high dose (20 mg/kg) showed signs of sedation 3–4 days p i

Potentiation of enhexymal Na anaesthesia in mice

Fig 11 compares the barbiturate potentiating effect of α-FPT, 2 HCl in aqueous solution with that of α-FPT-D in oil A clear-cut potentiation was demonstrated with α-FPT, 2 HCl in doses of 0 625 and 1 25 mg/kg, a maximal effect being obtained ½ to 1 hr after drug administration Only

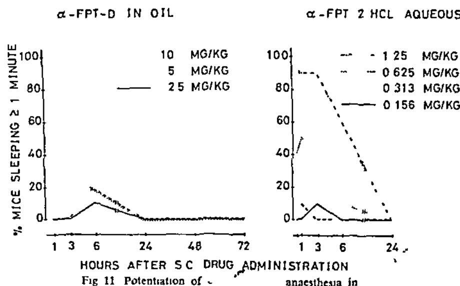


Fig 11 Potentiation of anaesthesia in

slight potentiation was obtained with α-FPT-D in oil, 2 5 to 10 mg/kg The effect of 10 mg/kg appeared 1 hr after injection and remained at the same level 3 and 6 hrs p i, while the effect of lower doses was maximal at 6 hrs Potentiation could in no case be demonstrated 24, 48 and 72 hrs p i The maximal effect with α-FPT-D in oil reached about the same level as that obtained with 0 3 mg/kg of α FPT, 2 HCl

Discussion

The comparison of the duration of pharmacological effects of FPT in the various dose forms has clearly demonstrated that dissolving FPT in an injectable oil prolongs the activity considerably By replacing α-FPT with the decanoic acid ester, which is several thousand times more lipophilic, the prolongation of the effect is even more pronounced This prolongation was clearly demonstrated with the anti-apomorphine-model in the dog and the CAR-model in the rat and mouse, where effects could be shown for up to 3 weeks, while in the anti-apomorphine-model in the rat the effect of even 8 mg/kg α FPT D in oil only lasted for 4 days This discrepancy between the two rat models seems to indicate that inhibition of CAR is a much more sensitive method for the detection of the central effect of FPT

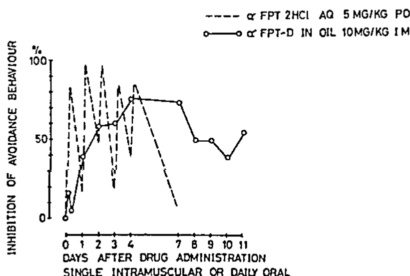


Fig 12. Comparison of inhibition of conditioned avoidance response after repeated oral administration of α FPT, 2 HCl aqueous solution, and intramuscular administration of α FPT D in oil

In fig 12 the inhibition curve of CAR for 10 mg/kg α-FPT-D in oil, injected intramuscularly has been superimposed on the curve representing the CAR inhibition obtained by repeated oral administration of 5 mg/kg α FPT, 2 HCl It can be seen that the intramuscular injection of 10 mg/kg α FPT-D in oil is roughly equivalent to a daily dose of 5 mg/kg α-FPT, 2 HCl orally, only that the effect builds up somewhat more gradually

The slow onset of action after α-FPT-D in oil seen in dogs and in the CAR experiments in rats probably reflects the slow release of active substance from the depot

In dogs the effect of α-FPT, 2 HCl and α FPT in oil sets in very rapidly and heavy sedation is seen, while with the depot preparation the antiemetic effect gradually increases up to the 7th day without any overt signs of sedation at any time This probably has some significance in terms of clinical use, since it might be said that the pronounced and relatively longlasting effect of 2 and 6 mg/kg of α FPT, 2 HCl in aqueous solution or α-FPT in oil is obtained at the expense of intolerable side effects, while with α FPT-D in oil the effect is smooth, longer lasting and without any side effects

α FPT D in oil caused a long lasting inhibition of CAR in rats while the cataleptic effect was rather weak and of considerably shorter duration Regarding clinical use these findings may indicate that extrapyramidal side effects after the injection of the depot preparation will be mild and transient and only occur at high dose levels several days after injection, which seems to be the case (ENERHEIM *et al* 1970)

The experiments on potentiation of barbiturate anaesthesia are important in order to assess whether potentiation of anaesthetics might be expected in case of a patient, under treatment with the depot preparation, has to undergo anaesthesia The potentiation observed after 10 mg/kg α FPT-D in oil was very modest and occurred only within the first 24 hrs These results should be seen in the light of the fact that in mice a very high degree of inhibition of CAR was obtained within the first 6 hrs after 10 mg/kg α-FPT D in oil, an effect which lasted for about 14 days It is unlikely, therefore, that any significant interference with an anaesthesia would occur in patients receiving the depot preparation

Thus the animal experiments have clearly demonstrated the prolonged neuroleptic effect of α FPT-D in oil Furthermore when the amount of active drug necessary to maintain neuroleptic effect with the depot preparation, is compared to the amount of orally administered drug necessary to maintain the same effect, a much better 'economy' of drug is achieved with the depot preparation The same experience has been gained from the clinical use of the depot preparation (GOTTFRIES 1971) An additional advantage of the depot preparation is the maintained smoothness of effect as compared with the serrated effect curve of orally administered FPT

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Characteristics of the "Receptor" for Narcotic Analgesics in Synaptic Plasma Membrane Fraction from Rat Brain

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Abstract The characteristics of the interaction between dihydromorphine (DHM) and a synaptic plasma membrane (SPM) fraction from rat brain cerebral cortex have been studied DHM at 10^{-9} M concentration is bound in a reversible process which is partly specific. The specific binding is inhibited by the sulph hydryl reagents, N ethylmaleimide and *p* chloromercuribenzoic acid The binding is strongly inhibited by 10^{-8} M levorphanol but significantly less inhibited by its optical antipode, dextrorphan, which is much less analgetic At 10^{-8} M concen tration, nalorphine and naloxone were strongly inhibitory while heroin at 10^{-8} M and codeine at 10^{-7} M were nearly inactive A mixture of 10^{-5} M acetylcholine and 2.5×10^{-5} M eserine caused a moderate inhibition while dopamine noradren aline, serotonin, histamine and propranolol were classified as inactive (i e caused less than 25 % inhibition at 10^{-5} M) The observed binding characteristics of the SPM fraction are compatible with those which might be expected for the actual narcotic receptor Analogous SPM preparations from subcortical parts of the cerebrum and from the brain stem also showed stereospecific binding of nar- cotics

Key words Narcotics – analgesics – brain – analgesic receptors

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It has recently been reported that a subcellular fraction enriched in synap- tic plasma membrane (SPM)[1] from the rat cerebral cortex stereospecifically binds DHM (TERENIUS 1973a) The specificity found is that which could be expected for the actual narcotic receptor This communication presents additional information on the characteristics of the DHM binding process

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[1] Abbreviations SPM synaptic plasma membrane DHM, dihydromorphine NEM N ethylmaleimide, PCMB *p* chloromercuribenzoic acid, d minute

Material and Methods

Drugs Labelled DHM (nominally 7 8-^{3}H) was prepared by catalytic tritiation of morphine base After removal of labile tritium the product was purified by repeated thin layer chromatography Purified samples were stored at −20° under nitrogen in absolute methanol The specific activity was 50 mci/mg and the radiochemical purity on thin layer chromatograms in several systems was better than 97 %

Naloxone was kindly donated by Dr H W Kosterlitz Department of Pharmacology University of Aberdeen Scotland levorphanol and dextrorphan were gifts from Ciba Geigy AG Basel Switzerland Dopamine and noradrenaline (noradrenalinum NFN) were gifts from Astra AB Sodertalje Sweden and propranolol was supplied by Scan meda Goteborg Sweden Acetylcholine eserine PCMB and NEM were purchased from Sigma Chemicals St Louis, Mo The other chemicals were obtained from the usual sources All chemicals used for the preparation of buffers etc were of reagent grade and the water was redistilled

SPM fraction The methods for the preparation of the SPM fraction is described briefly (TERENIUS 1973a) Brains were obtained from female Sprague Dawley rats weighing 125–150 g The animals were bled under ether anaesthesia and the whole brain was placed in ice-cold 0 32 M sucrose All operations from this step were carried out at 0–4° The cerebral cortical grey matter (in a few experiments subcortical areas or brain stem) was isolated homogenized and subjected to differential centrifugation essentially according to GRAY & WHITTAKER (1962) The crude mitochondrial fraction from the centrifugation was shocked osmotically (WHITTAKER *et al* 1964) and was centrifuged at 12,000 × g_{max} for 20 min The supernatant was carefully decanted and layered over a discontinuous sucrose gradient consisting of equal volumes of 0 4 1 0 and 1 2 M sucrose which had been allowed to pre equilibrate for 1 hour This was followed by centrifugation at 100 000 × g_{max} for 80 min The band between 0 4 and 1 0 M sucrose was designated the SPM fraction and was sampled for incubation In almost all experiments incubations (see below) followed immediately after the frac tionation A few experiments however were carried out on fractions previously frozen and stored at −70° Frozen preparations have the same binding capacity as before the freezing (TERENIUS unpublished results)

Incubations Aliquots of the SPM fraction were incubated at 25° with shaking in 4 ml of the following buffer (NaCl 124 mM KCl 5 mM KH_2PO_4 1.2 mM $CaCl_2$ 0 75 mM N 2 hydroxyethylpiperazine N 2 ethanesulfonic acid "HEPES" 26 mM pH 8 0 at 25° TERENIUS 1973b) If not stated otherwise in the text the following incubation procedure was followed After 10 min of pre incubation of the SPM fraction with or without non labelled inhibitors labelled DHM was added in 0 2 ml buffer to a final concentration of 0 8 × 10^{-9} M and incubation continued for 60 min The flasks were cooled on ice and the incubates were spun down at 120 000 × g_{max} for 30 min Aliquots of the supernatant were saved for radioactivity measurements and the remainder was discarded The pellets were rapidly washed with cold buffer The concentration of radioactivity of the supernatants and of the pellets (after digestion with soluene™ Packard Instrument Co) was measured by liquid scintillation The protein content was measured according to LOWRY *et al* (1951) with serum albumin as standard The binding of label to the pellets was finally calculated as d p m./mg protein

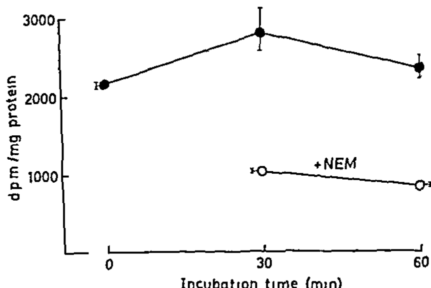


Fig 1 Typical time course of the binding of DHM to the SPM fraction and the effect of NEM on binding The SPM fraction was pre incubated in the buffer for 10 min at 25° After this DHM-³H and NEM were added The flasks were then either immediately chilled on ice and centrifuged or incubated for 30 or 60 min before centrifugation Each point represents the mean of 3 samples, the horizontal lines indicate the range The final concentration of DHM was 0 8 × 10^{-9} M, and of NEM 10^{-3} M

Results

In the previous communication (TERENIUS 1973a) incubation of the SPM fraction with DHM ³H was carried out for 60 min at 25° However, as can be seen in fig 1, the binding process is very rapid and no marked difference is found between the different incubation periods If the SPM fraction is treated with the sulphydryl reagent, NEM, no effect can be seen with competitors such as levomethadone (TERENIUS 1973a) or levorphanol (not shown) indicating that NEM inactivates the specific binding sites In fig 1, experiments with NEM were included in order to measure the specific part of the total binding (residual binding after NEM treatment considered as *nonspecific) The inhibition caused by NEM is about 60 % after 30* or 60 min incubation (see also table 3) Consequently, the specific part of the total binding is also about 60 % In all the following experiments an incubation period of 60 min was used in order to ensure equilibrium conditions

The binding process is reversible (table 1) since it is equally reduced by the addition of non labelled levomethadone before labelled DHM as well

Table 1

Reversibility of the binding of DHM-^{3}H at 0.8 × 10^{-9} M to the SPM fraction. The standard incubation procedure of 10 min pre incubation and 60 min incubation with DHM-^{3}H was followed. Levomethadone (at 2 × 10^{-8} M) was either present for 10 + 60 min or only for the last 30 min. NEM was added (to make a 10^{-3} M solution) after the 60 min. incubation with DHM-^{3}H and incubation was continued for 10 min. *Means of duplicates or triplicates (d.p.m./mg protein) are given. Each experiment was* run with a separate SPM preparation

Addition	Expt 1	Expt 2	Expt 3	Expt 4
None	2340	3110	3180	3000
Levomethadone	1430	1610	2050	1500
Levomethadone 30 min after DHM-^{3}H	1410	1630	1670	1570
	Expt 5	Expt 6	Expt 7	Expt 8
None	2560	3390	3620	3952
NEM 60 min after DHM-^{3}H	1170	2060	1520	1806

as 30 min after the addition of label. The binding is also strongly reduced if NEM is added to a SPM preparation which has previously been allowed to bind DHM-^{3}H.

The binding characteristics of the SPM fraction showed at least qualitatively the specificity which could be anticipated for the actual narcotic receptor (table 2). The potent analgesic, levorphanol, had a higher competitive affinity for the SPM fraction than its optical antipode dextrorphan, which is a much less active analgesic (cf. BECKETT & CASY 1962). The difference in inhibitory activity between the two antipodes in 5 experiments, including those shown in table 2, was highly significant ($P < 0.001$ in Student's t test). Codeine was inactive in the binding test while the two narcotic antagonists nalorphine and naloxone, inhibited the binding effectively. Heroin was not very active.

A number of non-analgesic compounds were tested for their effect on the binding of DHM. The concentration of these compounds was 1000 times that for the analgesics and analgesic antagonists tested. Only acetylcholine and eserine combined inhibited binding while dopamine, noradrenaline, serotonin, histamine and propranolol were listed as inactive since they produced less than 25 % inhibition in 3 experiments (these negative results are not shown in table 2).

The sulphydryl reagents NEM and PCMB, markedly inhibited binding at 10^{-3} M (table 2).

Table 2

Inhibition of the binding of DHM-^{3}H at 0.8×10^{-9} M to the SPM fraction · different experiments were run with different SPM preparations, in addition, all co pounds in each experiment were not tested with the same SPM preparation Howe the optical antipodes, levorphanol/dextrorphan were always tested with the sa preparation Mean values from duplicates or triplicates (d p m./mg protein) are show

Substance	Concentration (M)	Inhibition of binding (%) Expt 1	Expt 2	Expt 3
Levorphanol	10^{-8}	48	51	46
Dextrorphan	10^{-8}	21	7	6
Codeine	10^{-7}	9	12	17
Heroin	10^{-8}	13	19	12
Nalorphine	10^{-8}	44	42	45
Naloxone	10^{-8}	45	49	51
Acetylcholine + Eserine	10^{-5}	34	33	40
PCMB	2.5×10^{-5}	63	71	73
NEM	10^{-3}	62	61	70

The question whether stereospecific binding of DHM-^{3}H could occur in other parts of the central nervous system than in the cerebral cortex was tested A very crude dissection of the brain was performed to isolate the subcortical part of the cerebrum ("subcortex") and the brain stem The same subcellular fractionation procedure as used for the cerebral cortex was followed These preparations looked less well defined in the sucrose

Table 3

Binding of DHM-^{3}H at 0.8×10^{-9} M to SPM fractions from various parts of rat brain Means of duplicates (d p m./mg protein) are given Each experiment was run with a different SPM preparation.

Competitor	Subcortex Expt 1	Expt 2	Expt 3	Expt 4	Brain stem Expt 5	Expt 6	Expt 7	Expt 8
None	3770	2210	3820	3890	3430	3950	3970	3890
Levomethadone (10^{-8} M)	2000	1540	2210	2280	2180	1850	2490	2480
Dextromethadone (10^{-8} M)	2710	2170	3020	3050	2960	3920	3410	3290

gradients and were probably less pure than that from the cerebral cortex In all experiments with these preparations (table 3) the binding values were as predicted if the receptor was present

Discussion

The present data and those presented earlier (TERENIUS 1973a) show that the binding of DHM to the SPM fraction exhibits a high degree of specificity The stereo-specificity (i e discrimination between the optical antipodes levomethadone/dextromethadone (TERENIUS 1973a) and levorphanol/dextrorphan, table 2) is that which could be expected for the analgetic receptor, if it is assumed that stereoselective events at the receptor level are responsible for the differences in analgesic activity This assumption is supported by numerous studies with chiral narcotic agents In all cases studied, differences in distribution, metabolism etc were not likely candidates for the observed differences in analgesic potencies (PORTOGHESE 1970) It is also reasonable to conclude that optical antipodes should have nearly equal access to the receptors as long as their distribution is governed by passive processes If agents with different physicochemical properties (other than chirality), are compared the distributional factors become important (cf APPELGREN & TERENIUS 1973) However, the fact that codeine has a very low affinity supports the receptor concept since it is also a weak analgesic and the low affinity of heroin is understandable, if one assumes that its acetyl groups have to be split off before it can exert its biological activity In fact, 6 acetylmorphine and morphine are rapidly formed from heroin in the blood and the brain (WAY 1968) Since nalorphine and naloxone are inhibitors, this suggests that they act as narcotic antagonists by receptor blockade

If the observed binding affinities of the morphine congeners are considered in structural terms it seems as if tertiary substitution of the nitrogen with methyl (levorphanol) or allyl (nalorphine and naloxone) is compatible with binding affinity The free phenolic hydroxyl seems essential (present in the strong competitors levorphanol, nalorphine and naloxone but not in heroin and codeine) The free hydroxyl in position 6 (as in nalorphine) can be absent (as in levorphanol and naloxone) with retention of binding affinity Similar results were obtained for the binding characteristics of a subcellular fraction from the guinea pig ileum (TERENIUS 1972 & 1973b) The inactivity of a number of unrelated neuro-active substances (table 2) also points to the specificity of the system studied Whether the inhibition by a high concentration of eserine + acetylcholine is relevant for the conditions obtainable *in vivo* is not known Propranolol was included since it has very recently been stated (GROSZ 1972) that "under both *in vitro* and *in vivo* conditions

propranolol inhibits the 'binding' by the brain of radioactive morphine" Furthermore this effect, "seemed to be confined largely to the cerebral cortex, and other areas of higher sensory function and had no obvious influence on the cerebellum or the brain stem" Since the statements cited above is the only information provided by the author, the reason for the discrepancy with the present results cannot be evaluated

The data in table 1 and the fact that almost all the radioactivity is extractable in acid alcohol (TERENIUS, unpublished results) indicate that the process studied can be characterized as a reversible non covalent binding The sensitivity of the binding process to sulphydryl inhibition suggests that the 'receptor' or its immediate environment is part of a protein containing essential sulphydryl groups *It is interesting to note that sulphydryl groups* which are essential or modify binding properties are also present in other drug 'receptors', viz, for steroid hormones (JENSEN *et al* 1967, TERENIUS 1967), for cholinergic agents (KARLIN 1969) and for adrenergic agents (LEFKOWITZ *et al* 1972)

The 'receptors' seem to be distributed over various levels in the central nervous system (table 3) This is not an unexpected finding since morphine like drugs probably affect pain and other CNS mechanisms at different brain levels

Experiments with DHM at different concentrations (TERENIUS, unpublished results) showed that the dissociation constant for the specific binding under present conditions is 3–5 $\times 10^{-9}$ M Assuming a yield of 'receptor' in the preparations of 10 % (which is the average yield of the plasma membrane marker, 5 nucleotidase), the concentration of 'receptor in cerebral cortical grey matter is about 10 pmol/g fresh weight This value can be compared with the concentration in rat brain of the extremely potent analgesic, etorphine at doses which produces analgesia (DOBBS 1968) One can calculate the approximate maximum concentration to be 2 5 pmol/g (of whole brain) after an ED_{50} dose This value fits reasonably well with the present data if it is assumed that most etorphine is bound to the binding sites described above

Acknowledgements

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The Effect of Dopamine and Noradrenaline Antagonists on Amphetamine Induced Locomotor Activity in Mice and Rats

By

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Abstract The effect of noradrenaline antagonists, aceperone, phenoxybenzamine and dihydroergotamine and neuroleptic drugs with dopamine receptor blocking properties, i e haloperidol, *perphenazine*, *trifluperazine*, *spiramide* and pimozide was tested on the locomotor and rearing activity induced by amphetamine, 2 5 mg/kg, in rats In general it was found that the neuroleptic drugs in very low doses, haloperidol, 0 10 mg/kg, perphenazine, 0 05 mg/kg, trifluperazine, 0 15 mg/kg, spiramide, 0 05 mg/kg and pimozide, 0 15 mg/kg produced complete inhibition of the amphetamine activities, whereas much higher doses of aceperone, 20 mg/kg and phenoxybenzamine, 20 mg/kg only produced partial antagonism Dihydroergotamine (20 mg/kg) produced no significant effect on the amphetamine locomotor and rearing activity In mice trifluperazine (0 2 and 0 4 mg/kg) produced a very marked inhibitory effect, whereas spiramide (0 15 and 0 20 mg/kg) produced a significant but short lasting effect on the locomotor activity after 4 and 8 mg/kg d amphetamine Aceperone (5 and 10 mg/kg) and phenoxybenzamine (10 mg/kg) also produced a strong antagonistic effect on the motility Furthermore α methyltyrosine (250 and 350 mg/kg), an inhibitor of the biosynthesis of dopamine and noradrenaline, produced complete inhibition, whereas FLA-63 (20 and 40 mg/kg), an inhibitor of the formation of noradrenaline produced partial inhibition In conclusion these results indicate that the locomotor effect in mice and rats after amphetamine is dependent on both dopaminergic and noradrenergic mechanisms However, dopamine may be regarded as most significant, since the amphetamine motility only seems possible in the presence of functional active dopamine receptors

Key words Locomotor activity – amphetamine – noradrenaline antagonists – dopamine antagonists – neuroleptic drugs

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Biochemical and pharmacological studies have shown that the central stimulant effects of amphetamine are dependent on an interaction with the catecholamines dopamine and noradrenaline (FUXE & UNGERSTEDT 1970, GLOWINSKI 1970, SCHEEL-KRÜGER 1971, 1972a & b, STOLK & RECH 1970). However, it is more difficult to establish the individual role of dopamine and noradrenaline in the amphetamine induced locomotor activity in mice and rats. It is well known that α-methyltyrosine, an inhibitor of the biosynthesis of dopamine and noradrenaline, inhibits the central stimulant effects of amphetamine including the locomotor effect (DOMINIC & MOORE 1969, RANDRUP & MUNKVAD 1966, SCHEEL-KRÜGER 1971, STOLK & RECH 1970, SVENSSON 1970, WEISSMAN *et al* 1966). Disulfiram and diethyldithiocarbamate, which inhibit the formation of noradrenaline from dopamine reduce the amphetamine locomotor activity in mice and rats (MAJ & PRZEGALINSKI 1967, PFEIFFER *et al* 1966, RANDRUP & SCHEEL-KRÜGER 1966). However, some doubts have been raised on the specificity of these two last mentioned drugs (AIGNER *et al* 1967, MOORE 1969, SVENSSON & WALDECK 1970). Recently SVENSSON (1970) has shown that FLA-63 (bis-(4 methyl-1-homopiperazinyl-thiocarbonyl) disulfide), a new and more specific inhibitor of the enzyme dopamine-β-hydroxylase, produces partial inhibition of the amphetamine locomotor activity in normal but has no effect in reserpine pretreated mice.

Based on catecholamine uptake studies with d- and l-amphetamine TAYLOR & SNYDER (1970 & 1971) suggested that noradrenaline selectively mediates the locomotor activity. However, several recent studies do not agree with TAYLOR & SNYDER on the reported difference between these amphetamine isomers on brain noradrenaline. In contrast it was found in biochemical and functional studies that d- and l amphetamine showed similarities in potency to noradrenaline but differences with regard to brain dopamine (FERRIS *et al* 1972, MAJ *et al* 1972a, SCHEEL-KRÜGER 1972a & b, SVENSSON 1971). The differences between d- and l-amphetamine in eliciting hypermotility may thus rather be related to their different effect on the central dopamine neurons (SVENSSON 1971, SCHEEL-KRÜGER 1972a & b).

In the present study the respective role of dopamine and noradrenaline underlying the amphetamine motility in mice and rats was analysed by means of noradrenaline receptor blocking drugs and various neuroleptic drugs with specific dopamine receptor blocking effect. Furthermore FLA-63 and α-methyltyrosine were included in the studies on mice.

Materials and Methods

Locomotor studies in mice

The experiments were performed on SPF female albino mice 19–22 g of the

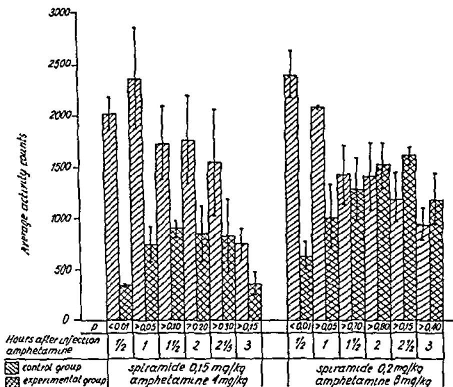


Fig 1 Effect of spiramide on the amphetamine induced hypermotility in mice

N M R I strain obtained from Dr Møllegaard s breeding center, Ejby L Skensved, Denmark Each mouse was only used once The experiments were performed between 8 a m and 4 p m at room temperature (19 21°)

Drugs

The following drugs were used d amphetamine sulphate, 4 and 8 mg/kg (Nordisk Droge), spiramide, 0 15 and 0.20 mg/kg (R 5808, Janssen Pharm) trifluperazine, 0 2 and 0 4 mg/kg (terfluzin®, Barker Rodhia), d,1 α methyltyrosinemethylester, 250 and 350 mg/kg (H 44/68, AB Hässle), phenoxybenzamine (bensylytum NFN), 10 mg/kg (blocadren®, A/S Alfred Benzon), aceperone, 5 and 10 mg/kg (R 3248, Janssen Pharm) and FLA-63, 20 and 40 mg/kg (AB Hässle) Aceperone d amphetamine, spiramide and trifluperazine were administered subcutaneously, whereas phenoxybenzamine H 44/68 and FLA-63 were administered intraperitoneally The injected volume was always 5 ml/kg Amphetamine was given 30 min after spiramide, trifluperazine and aceperone and 3 hrs after the injection of H 44/68, FLA-63 and phenoxybenzamine

The locomotor activity shown by 4 mice treated with antagonistic drugs plus amphet amine as against the control groups of 4 mice treated with amphetamine was measured simultaneously by two Animex activity meters (SVENSSON & THIEME 1969) The mice were placed in the plastic cages of the Animex 1 hr before the amphetamine injection, for acclimatization After 30 min the mice received the injection of compounds under

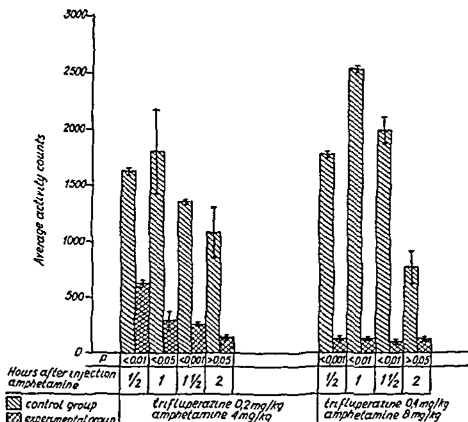


Fig 2 Effect of trifluperazine on the amphetamine induced hypermotility in mice

study or saline and were replaced in the cages Following another 30 min amphetamine was injected and the recording of the locomotor activity started immediately The activity counts were accumulated and recorded for each 30 min during continuous periods of 2 or 3 hrs Each drug combination of antagonists plus amphetamine was tested simultaneously with the corresponding amphetamine control group in three repeated schedules (n=3) The statistical significance was determined by Student s "t" test

Locomotor studies in rats

Male SPF Wistar rats obtained from Dr Møllegaard s breeding centre Ejby L. Skensved Denmark weighing 225–275 g were used All rats were kept in individual cages of wire netting (floor area 21 × 27 cm height 16 cm) at a room temperature of 21–22° The rats were placed in their cages 18 20 hrs before the experiments

Drugs

The following drugs were used d amphetamine sulphate 2.5 mg/kg (Nordisk Droge) aceperone, 10 and 20 mg/kg (R 3248) Janssen Pharm) phenoxybenzamine 10 and 20 mg/kg (blocadren®, A/S Alfred Benzon) d hydroergotamine 10 and 20 mg/kg

Table 1

Effect of various noradrenaline receptor blocking drugs on the locomotor and rearing activities induced in rats by 2 5 mg/kg d amphetamine The rats were pretreated with aceperone and dihydroergotamine 1 hr and phenoxybenzamine 3 hrs before amphetamine The peak effects of locomotor and rearing activities were classified according to the following scale (+) = non existent or very infrequent, + = weak and infrequent +(+) = present in short lasting continuous periods ++ = continuous but moderate in intensity and frequency +++ = continuous and very marked in intensity and frequency

Pretreatment	Number of rats	Time intervals	Number of rats in each behavioural activity category									
			Rearing					Locomotion				
			(+)	+	+(+)	++	+++	(+)	+	+(+)	++	+++
Saline	25	0 – ½ hr			25					25		
		½ – 1 hr					25					25
		1 – 1½ hr					25				25	
		1½ – 2 hr				25					25	
Phenoxybenzamine (20 mg/kg)	15	0 – ½ hr	15					15				
		½ – 1 hr	15					15				
		1 – 1½ hr	6	9				6	6	3		
		1½ – 2 hr	7	5	3			10		5		
Aceperone (20 mg/kg)	15	0 – ½ hr	15					15				
		½ – 1 hr	7	2	6			7			8	
		1 – 1½ hr	7	5		3		7			8	
		1½ – 2 hr	7	5		3		7	5		3	
Dihydroergotamine (20 mg/kg)	15	0 – ½ hr	1	14				1	14			
		½ – 1 hr	1		14			1				14
		1 – 1½ hr		1		14					15	
		1½ – 2 hr		1		14					15	

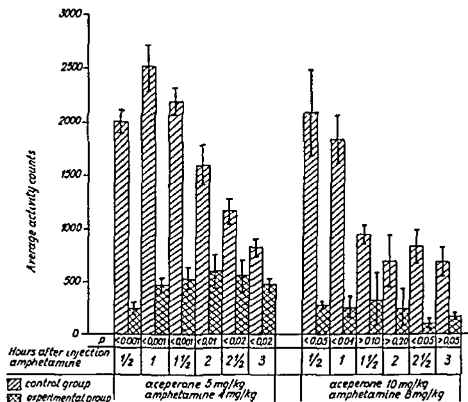


Fig 3 Effect of aceperone on the amphetamine-induced hypermotility in mice

(Sandoz), haloperidol, 0 05 and 0 10 mg/kg (serenase®, Janssen Pharm), perphenazine, 0 05 mg/kg (trilafon®, Schering), trifluperazine, 0 15 mg/kg (terfluzin®, Barker Rodhia), spiramide, 0 05 mg/kg (R 5808, Janssen Pharm) and pimozide, 0 05, 0 10 and 0 15 mg/kg (R 6238, Janssen Pharm) The neuroleptic drugs, haloperidol perphenazine, trifluperazine and spiramide were injected 30 min. before amphetamine Pimozide was given 3 hrs before amphetamine The noradrenaline receptor blocking drugs, aceperone and dihydroergotamine were given 1 hr before amphetamine, whereas phenoxybenzamine was injected 3 hrs before amphetamine All drugs were injected subcutaneously with the only exception of phenoxybenzamine, which was given intraperitoneally

The locomotor and rearing activity (standing up on the hind legs) of the rats was estimated semi-quantitatively according to the following scale

(+) = the activity is non existent or very infrequent
\+ = weak and infrequent
+(+) = present in short-lasting continuous periods (2–4 min)
++ = continuous but moderate in intensity and frequency
+++ = continuous and very marked in intensity and frequency

As a rule the rats were observed continuously under blind conditions during 2–3 hrs after amphetamine

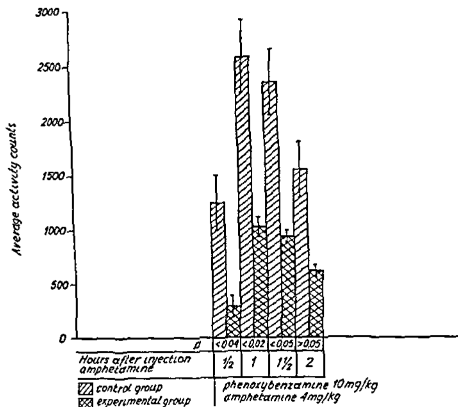


Fig 4 Effect of phenoxybenzamine on the amphetamine induced hypermotility in mice

Results

The amphetamine induced motility in mice

The results presented in fig 1 show that spiramide reduced the amphetamine-induced motility The effect of spiramide was short-lasting, since the antagonistic effect was most marked during the first half hour

Trifluperazine (fig 2) decreased the locomotor activity more markedly than spiramide The amphetamine antagonistic effect was long lasting and present during the 2 hours' recording period

The two noradrenaline receptor blocking drugs aceperone and phenoxybenzamine (figs 3 and 4) also reduced the amphetamine motility The effect of aceperone was long-lasting and more potent than phenoxybenzamine

α-Methyltyrosine (fig 5) antagonized nearly all locomotor activities, whereas FLA 63 (fig 6) only produced partial but significant inhibition of the amphetamine-induced motility

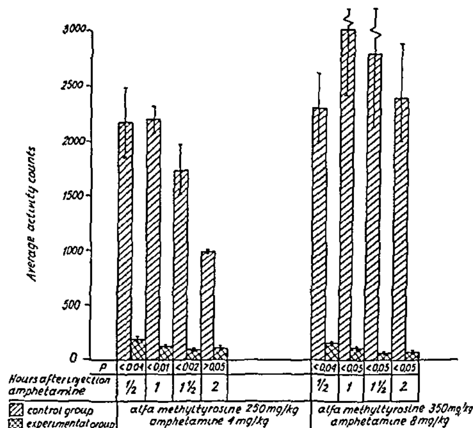


Fig 5 Effect of α methyltyrosine (H 44/68) on the amphetamine induced hyper motility in mice

The effect of noradrenaline receptor blocking drugs on the amphetamine-induced locomotor and rearing activity in rats

In previous studies from this laboratory (SCHEEL-KRÜGER 1971, 1972a & b) various doses of amphetamine were studied on the locomotor and rearing activities in rats Amphetamine in a dose of 2 5 mg/kg subcutaneously was found to produce a very marked and highly reproducible locomotor and rearing activity without the presence of extreme stereotyped activities such as licking and gnawing activities These activities are present in our rats weighing 200–275 g after doses of 5–10 mg/kg subcutaneously

The effects of noradrenaline antagonistic drugs presented in table 1 demonstrate that aceperone and phenoxybenzamine produced a marked inhibition of the locomotor and rearing activity induced by amphetamine (2.5 mg/kg)

Table 2

Effect of various neuroleptic drugs on the locomotor and rearing activities induced in rats by 2.5 mg/kg d amphetamine. The rats were pretreated with neuroleptic drugs ½ h before amphetamine. *However pimozide was given 3 hrs before amphetamine.* The peak effects of locomotor and rearing activities were classified according to the following scale (+) = non existent or very infrequent, + = weak and infrequent, +(+) = present in short lasting continuous periods, ++ = continuous but moderate in intensity and frequency, +++ = continuous and very marked in intensity and frequency

Pretreatment	Number of rats	Time intervals	Number of rats in each behavioural activity category									
			Rearing					*Locomotion*				
			(+)	+	+(+)	++	+++	(+)	+	+(+)	++	+++
Saline	25	0 – ½ hr			25					25		
		½ – 1 hr					25					25
		1 – 1½ hr					25				25	
		1½ – 2 hr				25					25	
Haloperidol (0.10 mg/kg)	10	0 – 3 hrs	10					10				
Perphenazine (0.05 mg/kg)	10	0 – 3 hrs	10					10				
Trifluperazine (0.15 mg/kg)	6	0 – 3 hrs	6					6				
Spiramide (0.05 mg/kg)	10	0 – 3 hrs	10					10				
Pimozide (0.15 mg/kg)	15	0 – 3 hrs	15					15				

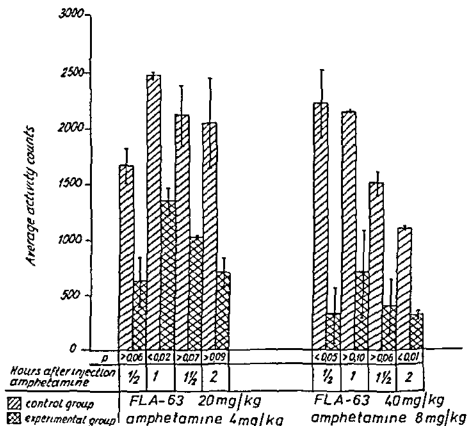


Fig 6 Effect of the dopamine β hydroxylase inhibitor (FLA-63) on the amphetamine hypermotility in mice

Phenoxybenzamine (20 mg/kg) was most active and produced complete inhibition during the first hour in all rats tested and partial inhibition during the following hour

Aceperone (20 mg kg) produced complete inhibition during the first 30 min and partial inhibition during the following 1½ hr

However, dihydroergotamine (20 mg/kg) produced only weak inhibitory effect on the amphetamine locomotor and rearing activity

None of these three tested drugs produced significant prolongation of the amphetamine stimulation which lasted 3–3½ hrs after 2 5 mg/kg amphetamine

During the preliminary studies *aceperone and phenoxybenzamine* were also tested with a lower dose of 10 mg/kg but the antagonistic effect on the amphetamine locomotor and rearing activities was much weaker than after the higher dose (20 mg/kg) of these drugs

The effect of neuroleptic drugs on the amphetamine locomotor and rearing activity in rats

In general the neuroleptic drugs were found to produce a nearly complete inhibition of the amphetamine locomotor and rearing activities with extremely small doses during the 3 hours' observation period (table 2) Only very infrequently did the rats move from one place of the cage to another Sometimes small nodding movements of the head were observed Grooming activities were completely absent in the control rats receiving only amphetamine (1–2½ hrs after the injection) but grooming spells mainly performed on the body with the head were sometimes observed after the combined treatment with neuroleptic drugs and amphetamine Most grooming spells were observed after the combined treatment of trifluperazine (0 15 mg/kg) and amphetamine (2 5 mg/kg)

The dose levels of the neuroleptic drug which produced complete inhibition of the amphetamine induced locomotor and rearing activities were as follows Haloperidol, 0 10 mg/kg, perphenazine, 0 05 mg/kg, trifluperazine, 0 15 mg/kg, spiramide, 0 05 mg/kg and pimozide, 0 15 mg/kg Haloperidol, 0 05 mg/kg and pimozide, 0 05, and 0 10 mg/kg also produced significant but only partial inhibition The other neuroleptic drugs have not been tested in lower doses

Discussion

The present study shows that the neuroleptic drugs, spiramide (0 15 and 0 20 mg/kg) and trifluperazine (0 20 and 0 40 mg/kg) given in low doses produce inhibition of the amphetamine (4 and 8 mg/kg) induced motility in mice Much higher doses of the noradrenaline receptor blocking drugs, aceperone (5 and 10 mg/kg) and phenoxybenzamine (10 mg/kg) are necessary to obtain significant inhibition

This result is in close agreement with the conclusion reached by VAN ROSSUM (1965, 1966 & 1970) on the amphetamine motility in mice

The present study extends this evidence to include the rat, in which species the amphetamine locomotor and rearing activities after 2 5 mg/kg are completely inhibited by the neuroleptic drugs, haloperidol (0 10 mg/kg), perphenazine (0 05 mg/kg) pimozide (0 15 mg/kg) spiramide (0 05 mg/kg) and trifluperazine (0 15 mg/kg) in extremely low doses

The noradrenaline antagonists, aceperone (20 mg/kg) and phenoxybenzamine (20 mg/kg) produce partial but marked inhibition in much higher doses

Dihydroergotamine (20 mg/kg) produces no or only weak inhibition which may be due to the lack of sufficient central potency (MUNOZ & C[illegible]STEIN 1961)

The results obtained with the inhibitor of dopamine and noradrenaline synthesis, α-methyltyrosine (H 44/68) and the noradrenaline synthesis inhibitor, FLA 63, on the mouse are in close agreement with SVENSSON (1970), since FLA-63 produces a partial inhibition, whereas α methyltyrosine produces complete inhibition

Other studies in this laboratory (AYHAN, unpublished results) have shown that FLA-63 produces a marked inhibition of the motility and rearing activity in rats following lower doses of amphetamine, but weaker effect against higher doses of amphetamine (see also CORRODI *et al* 1970)

The available evidence thus suggests that the amphetamine-induced motility is dependent on both dopamine and noradrenaline However, in reserpinized mice and rats the amphetamine-induced motility seems to be exclusively dependent on dopamine (SVENSSON 1970, SCHEEL-KRUGER 1971 & 1972b) The role of noradrenaline is supported by the results with inhibitors of noradrenaline formation and the noradrenaline receptor blocking drugs aceperone and phenoxybenzamine (see also Introduction) Pharmacological and biochemical studies have provided evidence which indicates that aceperone and phenoxybenzamine do not block dopamine receptors (ANDÉN *et al* 1964 & 1970a, CARLSSON & LINDQVIST 1963, JANSSEN *et al* 1965 & 1967, O KEEFFE *et al* 1970, SCHEEL-KRUGER 1972c)

However, it seems reasonable to conclude that dopamine may be regarded as more significant than noradrenaline since the motility only seems possible in the presence of functionally active dopamine receptors The present administered neuroleptic drugs have thus been selected as being relatively specific dopamine antagonists with no or only negligible effect on noradrenaline particularly in view of the low dose levels of these drugs used (ANDÉN *et al* 1970a & 1972, FOG *et al* 1968, JANSSEN *et al* 1965 & 1967, PEDERSEN & CHRISTENSEN 1972 RANDRUP & MUNKVAD 1970, VAN ROSSUM 1965 & 1966)

Previous studies with apomorphine and clonidine, drugs which stimulate directly dopamine and noradrenaline receptors respectively, have led to the hypothesis that the stimulation of locomotor activity requires the intact function of dopamine and is dependent on both dopamine and noradrenaline receptor stimulation (ANDÉN *et al* 1970b, FUXE & UNGERSTEDT 1970, MAJ *et al* 1972b) However, the behavioural excitation is changed from locomotor activity to stereotyped behaviour if the degree of dopamine receptor stimulation is too intense or the ratio of dopamine to noradrenaline receptor activity is increased above a certain level (ANDÉN *et al* 1970b, FUXE & UNGERSTEDT 1970) Similar conclusions also seem to apply to the amphetamine stimulation in the rat (see also CORRODI *et al* 1970) The locomotor exploratory stimulation is thus mostly characteristic following lower doses of amphetamine (1–2 5 mg/kg) Following higher doses (5–10 mg/kg) the loco-

motor and rearing activities in the rat are only present during the prephase to the performance of an extreme stereotyped activity consisting of continuous sniffing, licking and gnawing the animals placing themselves in a small area at the bottom of the cage Forward locomotion is absent during this stereotyped phase but backwards locomotion may occasionally be seen (RANDRUP *et al* 1963, ARNFRED & RANDRUP 1968, FOG 1969, SCHEEL-KRUGER 1971 & 1972a) Considerable evidence has shown that this stereotyped behavioural excitation is exclusively dependent on dopamine even in the absence of noradrenaline (RANDRUP & SCHEEL KRUGER 1966, SCHEEL-KRUGER & RANDRUP 1967, SCHEEL-KRUGER 1971, 1972a & b)

Acknowledgement

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On the Effect of a Polypeptide Isolated from "Kalata-Kalata" (*Oldenlandia affinis DC*) on the Oestrogen Dominated Uterus

By

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Abstract In African folk medicine an aqueous extract of *Oldenlandia affinis DC*, Kalata Kalata", is used to accelerate childbirth From the extract, which *induced strong contractions in isolated uteri* serotonin and an *utero-active* polypeptide have been isolated The peptide has been examined on oestrogen dominated uteri from rats and rabbits *in vivo* and *in vitro* and also on human uterine strips The oxytocic activity showed the same threshold value of 20 µg/ml *in vitro* for all three species *In vivo* the same dose caused ventricular fibrillation

Key words *Oldenlandia affinis DC* – oxytocic effect – cardiotoxic effect

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Aqueous extracts of the dried herb 'Kalata Kalata", *Oldenlandia affinis DC*, are extensively used by the natives in the Kasai Province of Zaire to accelerate childbirth (Gran 1970) Due to its oxytocic activity this drug is also used in the Central African Republic (Sandberg 1965) Aqueous plant extracts have been found to contract isolated rat uteri as well as strips from pregnant, human uteri (Gran 1970) Two utero active substances have been isolated from the drug i e serotonin and a polypeptide with a molecular weight of about 4000 (Gran 1973a) In the present work the uterine activity of this unknown peptide was examined *in vitro* and *in vivo* in rats and rabbits and in strips from the human uterus

Material and Methods

In vitro studies

1 Rats Uteri from Wistar rats (180–220 g) were suspended in aerated de Jalons solution (Gaddum *et al* 1949) maintained at 28° The rats were injected subcutaneously with stilboestrol (diaethylstilboestrolum NFN) (0 1 mg/kg) two days before they were killed and the uterine horns were used immediately The contractions were recorded

isotonically (Harward heart/smooth muscle recorder) The load was 1 to 2 g, and the recording was started when a constant response had been obtained with a standard, usually with oxytocin The contact time was adjusted to maximal contraction, and the uterus was washed at least four times and allowed 3 min relaxation before the next stimulation

2 Rabbits The method described by NESHEIM (1972) was used Circular and longitudinal strips were suspended in the same bath which was kept at 41° and oxygenated with 5 % CO_2 in O The strips were stretched to a mean tension of 1 g and were allowed to adapt for at least 1 hr before the addition of drugs The uterine tension was recorded *isometrically* Spontaneous activity was present in all preparations A cumulative administration of drugs was made at 5 min intervals with no washing out since this procedure disturbs the pattern of spontaneous activity

3 Human uterine strips Strips longitudinally excised from the fundus during Caesarean sections at or near term, were prepared and examined according to the method described by SULLIVAN & MARSHALL (1970) The strips (30×3×3 mm) were suspended in the oxygenated bath at 37° and kept under a tension of 2 g The contractions were *isometrically* recorded with Statham transducers and drugs were cumulatively administered

In vivo studies

1 Rats Adult Wistar rats (200–280 g) were selected in the pro oestrous phase according to their vaginal smears, anaesthetized by subcutaneous injections of urethane (7 ml/kg of a 25 % w/v solution) and tracheotomized A carotid artery was cannulated for registration of the blood pressure and a jugular vein for the intravenous administration of drugs The method of PICKLES & FITZPATRICK (1966) for recording the intraluminal, uterine pressure was used A 2 cm long uterine segment was isolated, and the intraluminal pressure was recorded by a Statham transducer (P23AC) connected to a Grass polygraph The blood pressure was recorded in the same way The temperature was kept at 35° by varying the distance of a heating lamp placed above the animal The volume of each intravenous injection was kept below 0 5 ml

2 Rabbits Oestrogenized rabbits (2 6 – 4 2 kg) treated like the animals used for the *in vitro* studies were anaesthetized with urethane (1 g/kg in 25 % w/v solution) given through an ear vein and a subcutaneous dose of 0 2 g/kg

Cannulation was made according to SETEKLEIV (1964) similar to the method used for the rats A 3 cm long uterine segment was isolated and filled up with 0 9 % saline under an initial pressure of 25 cm of water In addition the respiration was recorded using a carbon dioxide analyser (Capnograph, Godart 146) which continuously aspirated air for analysis through a needle inserted into the tracheal tube The temperature was kept at 38 5° by a rectal thermocouple connected to a thermo-regulated heater on the operation table Injected volumes of intravenously given drugs never exceeded 3 ml

Intragastric administration Drugs were instilled into the stomach through a gastric tube in 4 rats and 4 rabbits The rats received 10 mg of Kalata peptide in 5 ml saline the rabbits 100 mg in 50 ml The animals were then observed for 1 hr For comparison aqueous plant extract and thereafter serotonin in corresponding doses and volumes were administered (1 kg dried plant 20 mg serotonin 1 g Kalata peptide)

Drugs were diluted in the same solvent in which they were administered The *Kalata peptide* was prepared by Sandoz Ltd, (Basle Switzerland) according to the method previously described (GRAN 1973b) and in addition purified by adsorption chromatography on silica gel and on acid aluminium oxide. *Serotonin standard* was prepared from Serotonin Creatinin Sulfat Komplex (Schuchardt, Munich Germany)

Oxytocin Syntocinon® (Sandoz Ltd, Basle) and *Methysergide* 1 methyl-2 bromolysergic acid diethylamide (from the same firm) *Progesterone* Primolut N® (Schering Ltd Berlin Germany) was shaken with de Jalons solution at 40° to give a homogeneous suspension of progesterone (20 µg/ml) stable at 28° (SULLIVAN 1966) *Crude drug extract* was prepared by extraction of finely ground plant material with boiling water for 30 min

Results

In vitro studies Fig 1 shows contractions of the isolated rat uterus caused by serotonin, Kalata peptide and crude drug extract Contractions induced by the crude drug were rather similar to those obtained with serotonin Contractions obtained with the peptide, however, showed another pattern They were slower, stepwise progressive and increased the uterine tone Fig 1 further shows that methysergide inhibited both the serotonin and the plant extract, but had no significant effect on the peptide-induced contractions

Fig 2 shows that 10 µg/ml of the Kalata peptide had an action on the isolated rat uterus roughly corresponding to 0 08 milliunits (mU) of oxytocin When the activity of the two drugs was compared in relation to weight, the following relationship was found Oxytocin/Kalata peptide ≈ 1/50000 (one unit of oxytocin calculated as 2 2 µg of pure synthetic oxytocin (STURMER 1968)

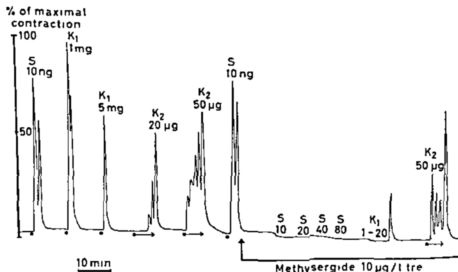


Fig 1 Isotonic contractions of the rat uterus due to stimulation by *serotonin* (S) in nanogram/ml *crude plant extract* (K_1) in mg plant/ml and *Kalata peptide* (K_2) in µg/ml of bath fluid before and after the addition of methysergide Maximal contraction the maximal response which could be produced with serotonin

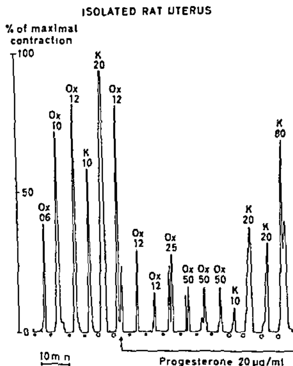


Fig 2 Isotonic uterine contractions after stimulation with *oxytocin* (Ox) mU/ml, and *Kalata peptide* (K) µg/ml of bath fluid before and after the addition of progesterone Maximal contraction the maximal response which could be produced with oxytocin

After the introduction of progesterone, the effect of oxytocin fell to a stable level within half an hour (same as SULLIVAN 1966) The Kalata peptide was also inhibited by progesterone (20 µg/ml), but not to the same extent as the oxytocin

The uterine reaction to Kalata peptide was also tested against other inhibitors, such as acetyl salicylic acid (40 µg/ml) known to antagonize prostaglandin provoked activity (VANE 1971), the antihistamine mepyramine maleate (0 2 µg/ml) and atropine sulphate (0 02 µg/ml) without showing any effect by these compounds

2 *Rabbits* The action of the Kalata peptide was different in the circulatory and longitudinally cut strips of the rabbit uterus In the circular strips a certain degree of inhibition could be observed, while a stimulating effect was found in the longitudinal muscles, fig 3 The doses required to provoke contractions of the rat uterus, (10 20 µg/ml), were also needed to initiate the effect on the rabbit uterus

3 *Human uterine strips* A stimulating effect of the spontaneous activity with an increase of the tone in the uterine strips was invariably found

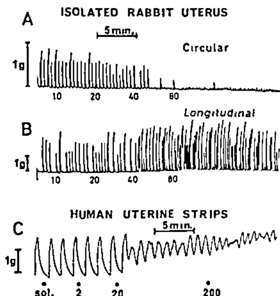


Fig 3 Isometric recordings of the uterine motility *in vitro* before and after the addition of *Kalata peptide* Figures indicate the doses added in µg/ml of bath fluid A circular muscle strips – inhibition, B longitudinal strips – excitation (both from rabbit) C human uterine strips – stimulation

This effect was seen with a dose of about 20 µg/ml or more, the same threshold dose as found for rats and rabbits

In vivo studies

1 Rats Urethane proved to be a stable anaesthetic A very small spontaneous uterine activity was seen when animals in pro-oestrus were selected Oxytocin induced dose dependent contractions when doses from 1 to 8 mU (4–32 mU/kg) were given

In a few rats a small stimulation of the uterine activity was observed after the injection of the Kalata peptide, but the contractions were inconstant, and no dose/response relationship could be found No uterine stimulation was induced in most of the animals (7 of 10), by intravenous doses from 1 to 100 µg of the peptide, (4–400 µg/kg)

When the Kalata peptide was injected into the peritoneal cavity, the conditions appeared similar to those found *in vitro* By means of an intraperitoneal catheter left in the wound, the drug which had been dissolved in 3 ml of 0 9 % saline at body temperature, was injected The threshold doses for obtaining uterine contractions, were the same as found in the *in vitro* tests, (about 20 µg/ml in the injected fluid) When normal saline alone was injected, no contractions occurred

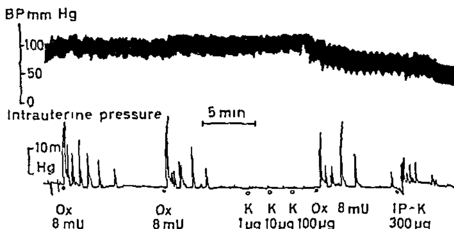


Fig 4 Intra luminal uterine pressure and blood pressure recorded in a rat (230 g urethane anaesthesia) during intravenous injections of *oxytocin* (Ox) in mU and of *Kalata peptide* (K) in µg At IP K the peptide was injected intraperitoneally in 3 ml of saline

In contrast to the weak uterine reaction on intravenous administration, a marked effect on the circulation was found ECG showed ventricular tachycardia, and the lethal dose was 1 mg/kg (0 6–2 0 mg/kg, mean 1 mg/kg, n 12) which resulted in ventricular fibrillation The arterial blood pressure was markedly decreased with doses of about 100 µg/kg, often preceeded by a short initial increase in blood pressure (fig 5)

2 *Rabbits* The effect of intravenous doses of the Kalata peptide was recorded in ten rabbits and proved to be rather similar to the action observed in the rats Doses of about 100 µg/kg induced uterine contractions (4 animals) Higher doses usually caused a fall in the blood pressure, and all the animals were killed by a dose of the peptide from 0 6 to 2 0 mg/kg (mean 1 2 mg/kg, n 10), the same lethal dose as found for the rats ECG recording during the injection of the lethal dose showed ectopic pacemaker and a final fibrillation End expiratory CO_2 percentage in the trachea during urethane anaesthesia was found to be 4 % (±1) After high doses of the Kalata peptide a hyperventilation was noted, and with lethal doses respiratory arrest followed after the circulatory collapse

Intragastric administration No uterine reaction and no circulatory effect due to intragastric administration of the drugs were recorded in the animals tested

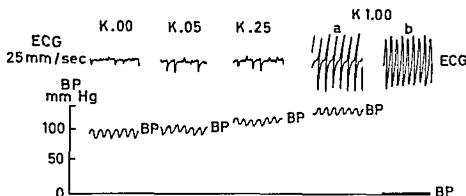


Fig 5 ECG and blood pressure variations in rat (220 g, urethane anaesthesia), during increasing intravenous doses of *Kalata peptide* (K) in mg/kg In relation to the last dose ventricular tachycardia and increased blood pressure are seen, immediately followed by ventricular fibrillation and exitus

Autopsy of all the animals was done immediately after cessation of the respiratory movements It showed in all cases a distended, fibrillating heart, distended central veins and a dark, bloodfilled liver Lung oedema was observed in 3 rabbits

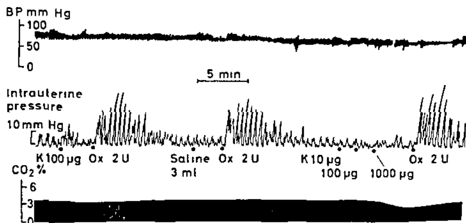


Fig 6 Blood pressure (BP) intraluminal uterine pressure and tracheal CO_2-concentration recorded from a rabbit (4 1 kg, urethane anaesthesia) during intravenous injections of *oxytocin* (Ox), *saline* and *Kalata peptide*, (K)

Discussion

The observation of an oxytocic activity following oral intake of the native drug lacks a scientific investigation and evaluation (SANDBERG 1965, GRAN 1970) The intensive use of the crude drug extract by two different and far separated tribes, probably through generations, makes it likely that some effect has been obtained from it

The phytochemical investigations on the plant revealed two utero-active substances, serotonin (20 mg/kg of dried herb) and a peptide (1 g/kg), (GRAN 1973a) The possibility of other uteroactive compounds present in the plant can not be excluded The recorded effect on isolated rat uteri caused by the crude drug, however, can fully be explained by the two isolated substances

Serotonin is known to cause uterine contractions both *in vitro* and *in vivo* when parenterally administered (SOUTHGATE & SANDLER 1968) Taken orally it is rapidly destroyed and thus can not be expected to cause uterine activity (GOODMAN & GILMAN 1970) This assumption was supported by the investigations performed, as neither the crude plant extract, nor serotonin provoked any activity in the animals after intragastric administration

The uterine activity due to the Kalata peptide observed *in vitro* and the absence of its effect *in vivo*, can most likely be explained by the marked cardiotoxicity of the peptide The concentration needed to provoke contractions *in vitro* (about 20 μg/ml), is practically identical with the concentration obtained in the blood after injection of an ordinary lethal dose (1 mg/kg body weight $\approx$ 20–30 μg/ml blood)

The high molecular weight of the peptide, about 4000, makes absorption from the gastrointestinal tract less likely Support for this assumption was given, as no physiological effect could be recorded after the intragastric administration of the drug

The pregnant, human uterus showed the same threshold value *in vitro* as the rat and the rabbit uterus An especially high sensitivity to the Kalata peptide of the human uterus should thus be excluded When the isolated peptide and oxytocin were alternatively injected intravenously during the animal experiments, no potentiation of the oxytocin activity was found This should exclude a possible synergism with the hormone

Serotonin has been shown to cause a toxic effect on the foetus (ROBSON & SULLIVAN 1968) As described above, the Kalata peptide also has a highly toxic effect when given parenterally A plant extract containing both these dangerous compounds can not be regarded as an obstetric therapeutic agent, and it should also be abandoned in primitive medicine, as complications have been reported to arise from the use of it (GRAN 1970)

The weak uterine activity compared to the high toxicity shown in animals,

does not seem to justify clinical trials with the genuine Kalata peptide Other peptides, present in small quantities, as well as degradation products might, however, be of interest for further investigations

Acknowledgements

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Effects of Chronic Ethanol Treatment on Rat Liver Lysosomes

By

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Abstract The subcellular distribution and the latency of the lysosomal enzymes b glucuronidase and acid DNase have been studied in livers from ethanol treated and control rats Some livers were isolated and perfused with or without ethanol Ethanol treatment (4–5 weeks) increased the free (non latent) lysosomal enzyme activity significantly without affecting the total activities In the perfused liver ethanol produced a small stabilizing effect on the lysosomes The lipid content of the liver did not change, and the plasma enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, b glucuronidase) and plasma bilirubin were in the present study unaffected by the ethanol treatment

Key words Lysosomal enzymes – ethanol – b glucuronidase – DNase

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Ethanol given to rats leads to increased serum levels of certain cytoplasmic enzymes (e g alanine aminotransferase and aspartate aminotransferase, for review, see FRENCH 1971) This is indicative of an increased fragility of the biological membranes and is consistant with the finding that liver mitochondria from ethanol treated rats show increased permeability *in vitro* (FRENCH & MORIN 1969) It seemed of interest to find out whether the lysosomal membrane is also influenced by ethanol treatment since it has been suggested that toxic agents cause tissue injury by inducing the release of acid hydrolases into the cytoplasm or by increasing the permeability of the lysosomal membrane (DE DUVE 1963) In the present study an attempt was made to evaluate the state of the liver lysosomes in rats exposed to chronic ethanol treatment We have, in addition, studied the effect of ethanol in the isolated perfused liver

Materials and Methods

Male Wistar rats weighing 275–300 g, were maintained on a fat rich diet (MØRLAND, 1973) Ethanol constituted about 30 % of the total calories consumed by one group of animals (ethanol treated animals), while sucrose replaced ethanol isocalorically in the control group The rats were treated for a period of between 4 and 5 weeks

Liver perfusions were performed as described previously (SEGLEN & JERVELL 1969) The livers were obtained from either ethanol treated or control rats Blood samples were obtained from male rats (400 g) fed on an ordinary laboratory diet 40 ml of perfusate (rat blood diluted with buffer to a final hematocrit of 18 %) was re-circulated through the liver at 37° and pH 7 4 The perfusion rate was between 1 5 and 2 0 ml/min./g liver After perfusion for 15 min ethanol was added as a single dose of 150 mg followed by a continuous infusion of approximately 120 mg per liver for 2 hrs In this way the perfusate ethanol concentration was kept at between 37 and 57 mM (0 17–0 26 %)

The animals were sacrificed by decapitation The liver was rapidly excised and chilled in ice-cold 0 25 M sucrose containing 1 mM Tris buffer, pH 7 30 Homogenization was done in a chilled Potter Elvehjem homogenizer with a teflon pestle rotating at about 1000 r p m The grinding consisted of 5 complete up and down runs with the pestle Special care was used to follow the same homogenization procedure to reduce the lysosome disruption

b Glucuronidase (GIANETTO & DE DUVE 1955) and acid DNase (DE DUVE *et al* 1955) were used as marker enzymes for hepatic lysosomes

To assess the latency of lysosomal enzymes we have either measured the b glucuronidase activity in the presence of 0.25 M sucrose (free activity) or acid DNase and b glucuronidase activity in a supernatant after high speed centrifugation of the homogenate (non sedimentable activity)

Bovine testes DNA (Sigma) and phenolphthalein glucuronic acid (Sigma) served as substrates for acid DNase and b-glucuronidase respectively Both enzymes were measured in the presence of 0 1 M acetate buffer, pH 5 0 The free activity of b glucuronidase (BERG & BIRD 1970) was measured in fresh homogenates and in the presence of 0 25 M sucrose and 0 05 M acetate buffer, pH 5 0 The incubation time was 10 min The total activities of both enzymes were measured after treating the homogenates with 0 05 % (v/v) Triton X 100 Since b glucuronidase is inhibited by sucrose (DE DUVE *et al* 1955), total activity was measured both in the presence and absence of sucrose to make comparisons between free and total activity possible It was found that the activity is reduced by about 27 % when sucrose was included in the assay mixture The amount of enzyme released from the lysosomes in fresh homogenates was measured in the supernatant after centrifugation of the homogenates in a Sorvall centrifuge for 3 000 000 g×min

Part of the b glucuronidase activity in liver homogenates is of microsomal origin (DE DUVE *et al* 1955) To detect possible differences between the subcellular distribution of b glucuronidase in ethanol treated and in control animals, complete differential centrifugation was carried out according to DE DUVE *et al* (1955)

Samples for the determination of plasma enzyme activities and bilirubin were withdrawn from the abdominal aorta while the animals were under light ether anaesthesia The blood samples were heparinized and then centrifuged for 10 min at 1500 r p m Bilirubin concentration was determined by autoanalyzer (Technicon method AA II 18) based on the method of JENDRASSIK & GRÓF (1938) Activities of the fol

lowing enzymes were determined in plasma b glucuronidase (FISHMAN 1955) alkaline phosphatase (BESSEY *et al* 1946) aspartate aminotransferase (GOT) (KARMEN 1955) and alanine aminotransferase (GPT) (WRÓBLEWSKI & LADUE 1956) All enzymes except b glucuronidase were measured automatically (LKB kinetic test recording initial re action velocity) based on the original methods

Proteins were measured according to LOWRY *et al* (1951)

Lipids were extracted by FOLCH s procedure (FOLCH *et al* 1957) from about 3 g of liver that had been stored in liquid nitrogen before use Triglycerides and phospholipids were separated by thin layer chromatography (SKIPSKI *et al* 1965) Triglycerides were then determined by the method of CARLSON & WADSTROM (1959) The phos pholipids were determined by phosphate analysis (FISKE & SUBBAROW 1925) after complete hydrolysis

Small samples of liver (about 200 mg) were fixed in 4 % formaldehyde stored in 60 % ethanol imbedded in paraffin sliced and stained and then examined in the light microscope

Results

Table 1 demonstrates that chronic ethanol treatment results in increased free activity of b-glucuronidase About 52 % of the enzyme is non latent in homogenates prepared from ethanol treated rats as compared with about 35 % in the controls There is no detectable difference in the total activities of b glucuronidase or acid DNase between the ethanol treated and control animals, whether the activity is expressed as activity per mg protein or activity per liver

Since microsomal b glucuronidase is non latent (DE DUVE *et al* 1955) it seemed possible that the large increase in free activity following ethanol treatment was due to a selective increase in a microsomal enzyme To determine the amount of b-glucuronidase of non-lysosomal origin, homogenates from ethanol treated animals and controls were fractionated by differential centrifugation The results of these experiments are demonstrated in fig 1 It can be seen that there is no significant difference in the relative amounts of microsomal enzyme in the experimental and control animals

The non sedimentable activities of b glucuronidase and acid DNase show minor but significant increases after ethanol treatment (table 1) Control values for b glucuronidase and acid DNase are 5 2 and 9 7 % of total activities respectively These activities are increased to 8 0 and 15 5 % of total activity for b-glucuronidase and acid DNase, respectively, after ethanol treatment (table 1) It can be seen that the non sedimentable activity of acid DNase is higher than that of b-glucuronidase both for the controls and experimental animals

Histological examination by light microscopy indicated that all the livers from the 7 control animals were normal A very slight cloudy swelling was

Table 1

Effects of ethanol treatment (4–5 weeks) on total, free and non sedimentable enzyme activities

	Ethanol treated		Controls		P
b glucuronidase, total activity	77 7 ± 6 5	(7)	88.2 ± 6.5	(7)	n s
Acid DNase, total activity[b]	1 87 ± 0 03	(3)	2 16 ± 0 03	(3)	n s
b glucuronidase, free activity[c] (%)	52 4 ± 2 8	(7)	34 7 ± 3 3	(7)	0 01
b glucuronidase, non sedimentable activity (%)	8 0 ± 0 7	(3)	5 2 ± 0 4	(3)	0 05
Acid DNase non sedimentable activity (%)	15 5 ± 2 2	(3)	9 7 ± 0 5	(3)	0 05
Body weights final (g)	291 ± 12	(7)	303 ± 16	(7)	n s
Liver weights, final (g)	11 0 ± 0 6	(7)	11 0 ± 0 5	(7)	n s
Protein content (mg/g liver)	223 ± 9	(7)	240 ± 9	(7)	n s

Values are means ± S E M Numbers in brackets indicate numbers of animals a Total activity is expressed as μg phenolphthalein/hr/mg protein at 37° b Total activity is expressed as o d /hr/mg protein at 37° c Free and non sedimentable activities are given as per cent of total activity

seen in two out of 7 livers from the ethanol treated group The remaining 5 livers appeared to be normal

The mean triglyceride content of livers analysed for lysosomal enzymes was 5 6 mg/g liver and 8 9 mg/g liver for ethanol treated and control animals, respectively, and no significant change in the phospholipid content was seen (The mean value for ethanol treated animals is 28 3 mg/g liver as compared to 26 8 mg/g liver in the controls) Thus the ethanol treated rats showed no sign of lipid accumulation

The data for plasma enzyme activities indicated that ethanol treatment had no significant effects on the activities of b glucuronidase or the other enzymes measured (see Methods) The bilirubin content of serum was also unaffected

Liver perfusion for 2 hours increased the free activity of b glucuronidase, particularly in the control livers (from 34 7 ± 3 3 to 50 2 ± 2 1 %) Ethanol added to the perfusate to a final concentration of 0 17–0 26 % was found to *reduce* free b-glucuronidase activity both in the controls and ethanol pretreated rats (from 53 8 ± 0 9 % to 48 2 ± 2 1 % in 5 livers, i e a significant ($P < 0\ 05$) reduction)

BERG & JANOFF 1968) It is therefore possible that b-glucuronidase is non latent while still inside the lysosome Ethanol does not seem to have any direct effect on the lysosomal membrane when tested on purified particles *in vitro* (LEE 1972) or in the perfused livers studied in the present experiment but the chronic *in vivo* effect may be caused by peroxidative decomposition of the structural lipids of the membrane, as ethanol has been shown to increase lipid peroxidation (COMPORTI *et al* 1967, DILUZIO 1963) This is, however doubted by other investigators (HASHIMOTO & RECKNAGEL 1968 FRENCH & MORIN 1969) Ethanol is known to increase the activity of microsomal hydroxylases and the amount of cytocrome P 450 (RUBIN *et al* 1968 RUBIN & LIEBER 1968) The possibility therefore remains that ethanol treatment may increase the oxidation of NADPH as a consequence of increased microsomal enzyme activity CHEN & McCAY (1972) recently showed that free radicals that were formed *in vitro* by the microsomal oxidation of NADPH could damage lysosomal membranes The labilization of lysosomal membranes would then be secondary effect of ethanol administration

FRENCH & MORIN (1969) found increased free activity of succinic dehydrogenase in rat liver mitochondria after chronic ethanol treatment. Such treatment has been shown by other investigators to reduce mitochondrial protein synthesis (RUBIN *et al* 1972) as well as general liver protein synthesis (BANKS *et al* 1970) Changes in lysosomal membrane proteins could also conceivably occur, and this could lead to changes in the membrane properties simil[illegible] those seen in the mitochondria

From the additional examinations for the absence of path[illegible] on the liver it is suggested that changes in the permeability membrane precede gross changes in liver composition [illegible] damage

PLATT & SCHNORR (1971) have demonstrated defects membrane in rat liver parenchymal cells after ethanol servation is consistent with the increased free b[illegible] present study However they found a decreased b-[illegible] both in the lysosomal fraction and in the lysosomal [illegible] enzyme activity was measured only in a postmitocho[illegible] natant after 80 000 g × min) which makes comp[illegible] [illegible]ge to the lysosomes might be so severe that a con[illegible] enzyme was lost from the liver Furthermore the e[illegible] intraperitoneally which may cause local damage to the [illegible] 1972)

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BERG & JANOFF 1968) It is therefore possible that b-glucuronidase is non latent while still inside the lysosome Ethanol does not seem to have any direct effect on the lysosomal membrane when tested on purified particles *in vitro* (LEE 1972) or in the perfused livers studied in the present experiment, but the chronic *in vivo* effect may be caused by peroxidative decomposition of the structural lipids of the membrane, as ethanol has been shown to increase lipid peroxidation (COMPORTI *et al* 1967, DILUZIO 1963) This is, however, doubted by other investigators (HASHIMOTO & RECKNAGEL 1968, FRENCH & MORIN 1969) Ethanol is known to increase the activity of microsomal hydroxylases and the amount of cytocrome P 450 (RUBIN *et al* 1968, RUBIN & LIEBER 1968) The possibility therefore remains that ethanol treatment may increase the oxidation of NADPH as a consequence of increased microsomal enzyme activity CHEN & McCAY (1972) recently showed that free radicals that were formed *in vitro* by the microsomal oxidation of NADPH could damage lysosomal membranes The labilization of lysosomal membranes would then be a secondary effect of ethanol administration

FRENCH & MORIN (1969) found increased free activity of succinic dehydrogenase in rat liver mitochondria after chronic ethanol treatment Such treatment has been shown by other investigators to reduce mitochondrial protein synthesis (RUBIN *et al* 1972) as well as general liver protein synthesis (BANKS *et al* 1970) Changes in lysosomal membrane proteins could also conceivably occur, and this could lead to changes in the membrane properties similar to those seen in the mitochondria

From the additional examinations for the absence of pathological effects on the liver it is suggested that changes in the permeability of the lysosomal membrane precede gross changes in liver composition and signs of cell damage

PLATT & SCHNORR (1971) have demonstrated defects in the lysosomal membrane in rat liver parenchymal cells after ethanol treatment This observation is consistent with the increased free b-glucuronidase found in the present study However they found a decreased b glucuronidase activity both in the lysosomal fraction and in the lysosomal supernatant, but the enzyme activity was measured only in a postmitochondrial fraction (supernatant after 80 000 g × min) which makes comparison difficult The damage to the lysosomes might be so severe that a considerable amount of enzyme was lost from the liver Furthermore the ethanol was administered intraperitoneally which may cause local damage to the liver (STRUBELT *et al* 1972)

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The *in Vivo* Effect of Tropolone on Noradrenaline Metabolism and Catechol-O-Methyl Transferase Activity in Tissues of the Rat

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Abstract Tropolone (100 mg/kg intraperitoneally) was given to rats and the catechol O methyl transferase (COMT) activity and tropolone concentrations were measured in the submaxillary gland and in the liver and the concentrations of noradrenaline (NA) and normetanephrine (NM) and the accumulation of metabolites from ^{14}C tyrosine were measured in the brain and in the submaxillary gland The maximal tropolone concentrations were found after 0 5 hr and were 50 % higher in the liver than in the gland After 2 5 hrs it was reduced to 70 % (liver) and 25 % (gland) Tropolone caused a slight reduction of 15 % in the COMT activity when measured *in vitro* but a rapid fall of 40–60 % was found in the NM concentrations After 24 hrs the NM concentration was normal in the brain but still maximally reduced in the gland A parallel decrease of the NA content was found in the brain but not in the gland The studies of metabolites from ^{14}C tyrosine indicated a reduced synthesis of NA from tropolone with no change in the NM fraction No significant traces of acidic or neutral metabolites from NA were found but there was evidence of the formation of transaminated products the most prominent of which was *p* hydroxy phenyllactic acid Two large radioactive metabolites could not be identified The results indicate that the measurement of COMT *in vitro* may not be an adequate measure of the enzyme inhibition *in vivo* The enzyme tropolone complex is probably easily released by the *in vitro* procedures

Key words Catechol O methyl transferase – tropolone – submaxillary gland – brain

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Tropolone is an inhibitor of catechol O methyl transferase (COMT) (BELLEAU & BURBA 1963) No evidence has been found for the *in vivo* O methylation of this compound Tropolone however, has the disadvantage that it is rapidly removed from the brain and moreover there are discrepancies in the results with regard to the action of tropolone on the formation of methylated metabolites from dopamine in the corpus striatum, the *in vitro*

inhibition of COMT and the action on the enzyme activity when determined in brain tissue from animals treated with tropolone (BROCH 1972) The reason for these discrepancies is probably the reversible binding of tropolone to the enzyme and the fact that the inhibitor is easily released

The present work deals with the action of tropolone as an inhibitor of COMT in the peripheral and central adrenergic nerves The following parameters were studied after single injections of tropolone in a fixed dose noradrenaline (NA) and normetanephrine (NM) concentrations and NA synthesis rate in the submaxillary gland and the brain, and COMT activities and tropolone concentrations in the submaxillary gland and the liver The NA synthesis rate was determined from the relative measurement of the amount of radioactive metabolites accumulated after an infusion of ^{14}C-tyrosine A qualitative study was also done on the acidic and neutral metabolites formed from ^{14}C-tyrosine

Materials and Methods

The experiments were performed as described in a previous work where experimental details are explained (BROCH 1972)

Animals and drugs

Albino Wistar rats of either sex were used in all experiments

Tropolone (Aldrich Chem Comp) was dissolved in distilled water 20 mg/ml and given intraperitoneally in a single injection of 100 mg/kg

^{14}C Tyrosine (U) (The Radiochemical Centre Amersham) specific activity 513 ci/mol was given by a constant intraperitoneal infusion over 20 minutes 50 μci per animal For experiments using two-dimensional chromatography the radioactive tyrosine was given in a single intraperitoneal injection of 50 μci 20 minutes before the animal was killed

Assays of amines

Three submaxillary glands or 2 forebrains were pooled for each determination The amines were acetylated extracted into dichloromethane and separated by paper chromatography before the spectrophotofluorimetric determinations (CRAWFORD & YATES 1970) All determinations were corrected for their recoveries which were (percentage means ± S D) for NA 91 ± 25 (10 estimates) and for NM 75 ± 14 (7 estimates)

Determination of metabolites from ^{14}C tyrosine

The amine metabolites were determined in the brain stem and the submaxillary gland The organs were dissected out immediately after the infusion of radioactive tyrosine The brain stem was cut at the level between the cerebral hemispheres and the cerebellum The hemispheres the hippocampi and the corpora striata were removed from the forebrain and the remainder was used for the determinations The brain stem from one animal was homogenized in 2 ml 0 4 N perchloric acid and the submaxillary glands in a volume of 4 ml After removal of the perchloric acid by precipitation as the potassium salt, the acidic and neutral metabolites were extracted into ether and the

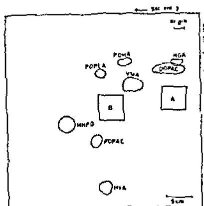


Fig 1 A typical two dimensional chromatogram with markers showing the places of possible tyrosine metabolites

DOPAC	=	3 4-dihydroxyphenylacetic acid
HGA	=	*homogentisic acid*
HVA	=	homovanillic acid
MHPG	=	3 methoxy-4-hydroxyphenylglycol
POMA	=	*p* hydroxymandelic acid
POPAC	=	*p* hydroxyphenylacetic acid
POPLA	=	*p* hydroxyphenyllactic acid
VMA	=	vanillylmandelic acid
A, B	=	unknown metabolites

amines remaining in the aqueous layer were acetylated and then extracted with dichloromethane and separated by paper chromatography according to CRAWFORD & YATES (1970) The radioactivity of the paper fractions was counted in a liquid scintil lator A sample of 0 1 ml of the extract was taken out for the determination of total activity before the extraction of acids

The acid fractions were separated by two dimensional descending paper chromato graphy (fig 1) Whole forebrains without corpora striata were homogenized in 4 ml 0 4 N perchloric acid After removal of perchloric acid the extracts were acidified with 0 1 ml 2 N HCl and extracted twice with 10 ml ether The ether extracts were com bined washed with 5 ml 0 1 N HCl, and the solvent was evaporated with a stream of nitrogen The residue was dissolved in a small amount of methanol and applied to a Whatman No 1 paper (46 × 46 cm) The chromatogram was developed for 5 hrs in system 1 (chloroform acetic acid water, 2 2 1 by vol) and then taken up, dried, turned 90° and run for 17 hrs overnight in system 2 (propanol ammonia water, 8 1 1 by vol) Markers were run simultaneously in each system Squares of 5 × 5 cm were cut ac cording to the markers and transferred to the scintillation vials containing a toluene based phosphor

The purity of the radioactive tyrosine was checked Five µl was dissolved in 2 ml water acidified with HCl and extracted with ether After washing the ether with 0 1 N HCl it was evaporated and the residue was dissolved and run on a two-dimen sional chromatogram No fraction showed an activity higher than 0 04 ‰ of the original tyrosine solution

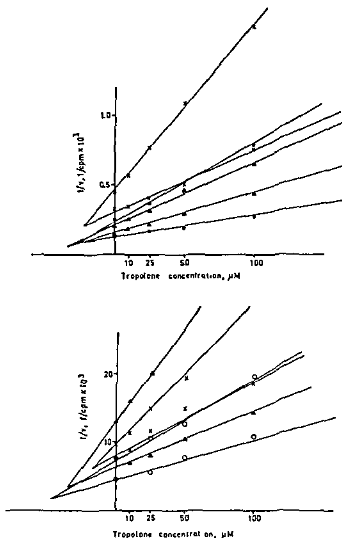


Fig 2 Determination of K_i for tropolone, a) in the liver and b) in the salivary gland Three independent experiments, each with two different concentrations of substrate, were done

Determination of tropolone

The tissues were homogenized in 0 4 N perchloric acid After removal of the perchlorate, the tropolone was extracted twice from a neutral supernatant with 10 ml chloroform After re extraction into 0 1 N NaOH and adjusting the pH to 6 0 the samples were read in a spectrophotofluorimeter at 310/400 nm The double extraction with chloroform gave a better recovery with smaller variations (69 % ± 15, S D – 6 estimations) compared to the earlier 50 % with a single extraction (Broch 1972)

COMT determination

A radioactive method was used (BROCH & FONNUM 1972) with a slight modification in that the concentration of coenzyme (SAMe) had to be increased to obtain optimal conditions for the tissues and the substrate concentration was also reduced. The volume of the incubation medium was 1 ml with the following solute concentrations 0 6 mM 3 4-dihydroxyphenylacetic acid 4 8 mM $MgCl_2$, 120 mg/liter SAMe (0.28 mM of a pure preparation) for the submaxillary gland and 360 mg/liter (0 84 mM) for the liver The tissues were homogenized in phosphate buffer pH 7 8 which was diluted to a final concentration of 0 04 N – ^3H (Methyl)-SAMe was added in tracer amounts The incubation time was 30 minutes

Monoamine oxidase determination

This was done in the brain stem according to SNYDER & HENDLEY (1968)

Enzyme kinetics

The inhibition constant of tropolone was determined by a method developed by Dixon (WEBB 1963) The tropolone concentration (abscissa) was plotted against the inverse value for the enzyme activity at two different substrate concentrations (0 3 and 0 6 mM) The abscissa of the intercept of the two lines gave the inhibition constant (K_i) The lines were constructed using the method of least sum of squares The Michaelis Menten constants (K_m) were determined from Lineweaver Burk plots Three experiments were performed for each constant

Results

Kinetic constants

The K_m values obtained using 3,4-dihydroxyphenylacetic acid as a substrate were (mean of 3 determinations ± S E M) $0 9 \pm 0 36 \times 10^{-4}$ M for the submaxillary gland and $1 9 \pm 0 94 \times 10^{-4}$ for the liver The corresponding K_i values for tropolone were (fig 2) $3 4 \pm 1 7 \times 10^{-5}$ M and $2 7 \pm 0 7 \times 10^{-5}$ M The intercepts of the curves were always found in the second quadrant of the coordinate system This pointed to a competitive inhibition although the nature could not be determined with certainty It has been shown for 4 methyltropolone using a more purified enzyme preparation that the inhibition is competitive (MAVRIDES *et al* 1963)

COMT activity and tropolone concentration (table 1)

Half an hour and one hour after tropolone (100 mg/kg intraperitoneally) the drug concentrations in the liver were comparable to those found for the brain and reported in an earlier investigation (BROCH 1972) except that the tropolone was retained for a longer time in the liver In the salivary gland the maximum concentrations were also found at 0 5 and 1 hr but only 60 % of the concentration in liver was reached There was still some tropolone in the liver 24 hrs after the injection

The COMT activity when measured *in vitro* after the *in vivo* administration of tropolone was reduced by 15 % in the two tissues This reduction

Table 1

Tropolone and amine concentrations and COMT activity in the rat brain, submaxillary gland and liver after tropolone, 100 mg/kg intraperitoneally Mean ± S E M (number of determinations)

Time hrs	Tropolone μg/g	COMT activity μmol/g/hr	Noradrenaline μg/g	Normetanephrine μg/g
		BRAIN †		
0	0	0 044±0 002	0 23±0 018 (5)	0 032±0 006 (5)
0 5	21 ±3 4			
1	19 ±5 1	0 032±0 005	0 19±0 024 (5)	0 019±0 006 (5)
2 5	3 5±1 0	0 034±0 004	0 13±0 014*(5)	0 014±0 004*(5)
24	0 3±0 2	0 040±0 008	0.22±0 022 (5)	0 046±0 005 (5)
		SUBMAXILLARY GLAND		
0	0	0 75 ±0 06 (6)	1 21±0 09 (10)	0 37 ±0 079 (6)
0 5	12 8±2 1 (6)			
1	11 4±1 9 (6)	0 73 ±0 04 (6)	1 07±0 11 (10)	0.28 ±0 066 (7)
2 5	4 4±0 9 (6)	0 63 ±0 05 (6)	1 05±0 15 (9)	0 14 ±0 026*(7)
24	0 3±0 1 (6)	0 74 ±0 07 (6)	1 56±0 09* (10)	0 15 ±0 037*(5)
		LIVER		
0	0	17 7 ± 0 3 (6)		
0 5	18 6±2 6 (6)			
1	19 5±1 4 (6)	16 7 ± 1 0 (6)		
2 5	12 3±2 3 (6)	14 6 ± 1 0* (6)		
24	1 5±0 6 (6)	17 9 ± 1 0 (6)		

* Significantly different from 0 hr $P < 0\ 05$ (t test)

† Values of tropolone from brain and COMT from brain stem *cit* BROCH (1972)

was significant for the liver only The maximal reduction was found at 2 5 hrs and this time did not correspond to the time for the maximal tropolone concentrations

In vivo inhibition of O methylation

The concentration of NM decreased significantly to the lowest value of 50 % of the control in the brain and 40 % of the control in the salivary gland 2 5 hrs after the injection of tropolone After 24 hrs the NM content of the brain was normalized while in the salivary gland it was still 40 % of normal (table 1) No significant change was seen in the NA concentration of the submaxillary gland, apart from a small increase after 24 hrs In the brain a decrease to 60 % of the normal was found, which parallelled the changes in the NM concentration

Table 2

Radioactive metabolites in the brain stem and submaxillary gland after tropolone, 100 mg/kg intraperitoneally, and constant intraperitoneal infusion of ^{14}C-tyrosine over 20 minutes Values in ‰ of total activity, mean of 4 experiments ± S E M

Hours after tropolone	Total activity cpm × 10^{-3}	Noradrenaline	Normetanephrine
	BRAIN STEM		
0	17±5 5	0 74±0 13	0 26±0 05
1	15±5 7	0 30±0 05*	0 20±0 09
2 5	21±4 2	0 23±0 05*	0 23±0 06
24	22±5 9	0 92±0 11	0 31±0 04
	SUBMAXILLARY GLAND		
0	46±8 7	0 54±0 08	0 28±0 03
1	33±5 5	0 13±0 06*	0 27±0 07
2 5	71±2 6*	0 24±0 05*	0 30±0 08
24	48±6 5	0 53±0 12	0 37±0 06

* Significantly different from 0 hr ($P < 0\,05$, *t* test)

Amine synthesis from ^{14}C-tyrosine

The radioactivity in the NA fraction was considerably reduced both in the submaxillary gland and in the brain stem at 1 and 2 5 hrs after the tropolone injection (table 2) returning to normal values after 24 hrs No change was found in the NM fractions These were quite small in the control animals but some decrease might have been detected With the method used it was impossible to separate octopamine from NM but it was considered unlikely that octopamine would be synthesized to a significant degree under the prevailing experimental conditions

Acidic and neutral metabolites of ^{14}C tyrosine

One dimensional chromatography was originally used for the separation of the acidic fractions as this was sufficient for dopamine metabolites A study of the NA metabolites after tropolone gave a non significant reduction of 50 % in the radioactivity of the "3-methoxy-4-hydroxyphenylglycol' and 'vanillylmandelic acid' fractions with large variations in the results By further separation of the fractions on the two-dimensional system it could be shown that the main activity in these fractions came from other substances which could not be separated adequately from the two O-methylated NA metabolites in solvent 1 (table 3, fig 1) The results from two-dimensional chromatography varied more than those from the one dimensional amine fractionation and required higher values could be accepted as significant. Activities up to 0 3 ‰

Table 3

Radioactive metabolites in brain and submaxillary gland after constant infusion of [14]C tyrosine over 20 minutes Values in ‰ of total radioactivity, mean of 3 experiments ± S E M

	p Hydr oxy phenyl acetic acid	*p* Hydr oxy phenyl-lactic acid	Methoxy-hydroxy phenyl glycol	*Vanillyl* mandelic acid	*p* Hydr oxy mandelic acid	A†	B†
			BRAIN				
Control	0 4±0 05	1 0±0 1	0 1±0 04	0 1, 0 1	0 3±0 1	2 6±0 3	4 1±0 7
Pargyline, 75 mg/kg	0 1±0 05*	1 9±0 6	0	0 1±0 07	0, 0	2.4±0 3	1 2±0 3*
			SUBMAXILLARY GLAND				
Control	0 6±0 4	0 5±0 1	0	0	0 2±0 08	7 8±1 8	0 7±0 4
Pargyline, 75 mg/kg	0 6±0 1	0 9±0 2	0 1±0 1	0 1±0 03	0 3±0 03	6 2±1 6	1 0±0 4

* Statistically different from control ($P < 0 05$)

† Unknown metabolites

doubtful This represented about 30 % of the blank values from chromatography paper

One of the identified metabolites was *p*-hydroxyphenyllactic acid Some activity was also found in the *p*-hydroxyphenylacetic acid fractions In the brain this fraction was reduced significantly by Pargyline while no change was seen in the gland On the other hand, the *p*-hydroxyphenyllactic acid fractions increased considerably in the brain with Pargyline The increase was not significant

The greatest activities were found in two fractions which were different from all usual aromatic tyrosine metabolites shown in fig 1 One of these metabolites ("B") was prominent in the brain only and it was reduced to 30 % by Pargyline treatment The other metabolite ("A") was found in appreciable amounts both in the brain and in the submaxillary gland and was not changed by Pargyline The *p*-hydroxyphenylpyruvic acid was unstable in both chromatographic solvents and could not be detected

No activities were found in the fractions of the methylated NA metabolites 3-methoxy-4 hydroxyphenylglycol and vanillylmandelic acid either in the brain or in the gland (table 3) Incubation with hydrolyzing enzymes (Glusulase, Endo) of the water residue from one of the brains after extraction of the acid metabolites gave no additional activity in the 3 methoxy-

Table 4

Monoamine oxidase activity in the brain stem 4 hrs after Pargyline 75 mg/kg intraperitoneally µmol/g/hr, mean of 4 determinations ± S E M

Control	12 2 ± 0 4
Pargyline	0 1 ± 0 002

4-hydroxyphenylglycol fraction However, the glycol was not stable in 0 4 N perchloric acid since it was converted to a substance with acidic properties showing red fluorescence in UV light By paper chromatography in solvent 1 it streaked from the positions of DOPAC to POPAC and in solvent 2 it was found between VMA and substance "A" (cfr fig 1)

Less than 1 per cent of the normal monoamine oxidase activity could be found in the brain stem after the Pargyline treatment (table 4)

Discussion

The present results show that tropolone is rapidly excreted from the organism The discrepancies in results between the *in vitro* inhibition of the enzyme and the inhibition of O methylation after the administration of tropolone to the animals indicate that tropolone is readily released from the tissues and the enzyme The maximal tropolone concentration in the submaxillary gland was about 0 1 mM, a value exceeding the K_i values by a factor of three The maximal decrease in the NM concentration was found 1 5 hrs later Hence there is a fair association in time between these parameters as some time is needed for the endogenous NM to be metabolized As the amount of tissue used for the COMT determinations was 18 mg per ml incubation medium, the concentration of tropolone in the medium can be calculated to be 1 7 µM at 1 hr and 0 7 µM at 2 5 hrs These values may represent quite well the small degree of inhibition of COMT measured after a tropolone injection Thus, tropolone is not firmly bound to the enzyme like, for instance, the monoamine oxidase inhibitors to the monoamine oxidase (ERWIN & DEITRICH 1971)

Infusion of radioactive tyrosine for the study of catecholamine synthesis and metabolism was not found suitable for the study of noradrenaline metabolites In the dopaminergic system of the corpus striatum the activity of tyrosine hydroxylase is especially high (McGEER *et al* 1967) and the turnover of dopamine is higher (half life 2 5 hrs IVERSEN & GLOWINSKI 1966, BRODIE *et al* 1966) than that of NA in peripheral nerves (life

8 3 hrs in rat heart COSTA *et al* 1966, SEDVALL *et al* 1968) with a half-life of NA in the central nervous system lying between these values (2 8–4 and 4 hrs BRODIE *et al* 1966, NEFF *et al* 1969) In the dopaminergic system of the striatum, activity was found not only in the amine fractions but also in the fractions of the two acid metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid (BROCH 1972) The main metabolite from NA in rat brain has been identified as 3 methoxy-4-hydroxyphenylglycol conjugated to sulphate (SCHANBERG *et al* 1968) The procedure used in the present work was not found to be suitable for the isolation of this metabolite even if hydrolysis of the conjugate was performed

Most of the tyrosine is, probably, metabolized through other pathways like transamination and incorporation into proteins The *p*-hydroxyphenylacetic acid was reduced significantly in the brain from traceable amounts to almost zero Two pathways are theoretically possible for this metabolite It may be the result of transamination of tyrosine to *p* hydroxyphenylpyruvic acid with subsequent decarboxylation Secondly tyrosine may be decarboxylated to tyramine and then deaminated A decrease in the *p*-hydroxyphenylacetic acid after Pargyline treatment would favour the latter hypothesis The decrease was seen in the brain only, and it was associated with a corresponding increase in the *p*-hydroxyphenyllactic acid, a second metabolite of *p*-hydroxyphenylpyruvic acid

It is most likely that Pargyline induced a shift in the metabolism of *p* hydroxyphenylpyruvic acid in the brain The *p*-hydroxyphenylpyruvic acid could not be detected because of its instability in both chromatographic solvents At any rate, the turnover of this acid is presumably so high that appreciable amounts do not accumulate in tissues

One of the unidentified metabolites, B, which was most prominent in the brain was reduced by 60 % by Pargyline It is not assumed, however, that it is a product of deamination as the reduction should then be expected to be larger, as the monoamine oxidase activity was almost completely inhibited

The reduction in the radioactivity of the NA fraction pointed to a reduced synthesis This could be the result of an inhibitory effect of tropolone on the synthesizing enzyme, e g. tyrosine hydroxylase (GOLDSTEIN *et al* 1967) This might especially be true for the brain as the NA content showed a clear reduction during the first 2 5 hrs In the submaxillary gland the NA concentration remained constant in spite of a rapid reduction of the synthesis indicated by the maximal reduction in radioactive NA after 1 hr This probably is the result of an inhibition of both tyrosine hydroxylase and COMT In the dopaminergic system an increase in the catecholamine concentration was found 1 hr after the tropolone injection (BROCH 1972) This indicates a smaller activity of tropolone on tyrosine hydroxylase in this system

The decrease in the NM content was much more prolonged in the submaxillary gland than in the brain. These differences probably reflect differences in the turnover rates of NA in the central and the peripheral nervous system.

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Morphine Analgesia in Rats at Various Ages

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Abstract Groups of 8–16 rats at various ages (20, 26, 32, 42 days old and adult animals) were injected subcutaneously with morphine and the degree of analgesia measured, using the hot plate technique, at 30, 60, 90, 120 and 150 min after the injection The rise and decline of analgesia was steeper in animals in the younger age groups than in the 42 days old or in the adult animals. The degree of analgesia was thus at a maximum at 30 or 60 min in the 20, 26 and 32 days old rats, whereas a peak analgesic effect was measured at 60 or 90 min. in the 42 days old and the adult animals Sensitivity to morphine analgesia was greatest in the youngest age group and then gradually declined towards the adult level When comparisons were made at the height of analgesia, 7,5–10 times greater doses were required in the adult animals in order to produce a degree of analgesia of the same order of magnitude as that obtained in the 20 days old animals At the age of 26 days the difference in sensitivity was about threefold and approximately twofold at the age of 32 days The analgesic effect was very similar in the 42 days old rats and in the adult animals The high degree of *sensitivity to morphine analgesia in the 20 days old animals was matched by* very high amounts of morphine in the brain 40 min after the subcutaneous injection of 5 mg/kg morphine labelled with ^{14}C in the aminomethyl group The amounts of morphine in the brain of the 26 days old rats were thus less than one half of that found in the brain of the youngest animals Brain levels of morphine in the 26 days old animals were significantly different from those in the brain of 42 days old and adult rats but not from those found in the brain of the 32 days old animals The amounts of morphine in the brain of the 32 days old animals did not differ significantly from that found in the 42 days old or in the adult animals The ratio between the amounts of morphine in plasma versus brain increased gradually with age (20 days old animals 1 77, adult animals 3 44) Accessability of morphine to the brain thus decreased with age by a factor of two It was accordingly concluded that the relatively lower amounts of morphine in the brain could to a certain extent, at any rate, explain the lower sensitivity in the older than in the younger rats The results also indicate that the sensitivity to morphine analgesia may decrease *per se* with age Analgesic experiments with young of mothers treated with morphine late in pregnancy showed that the highest degree of analgesia obtained was the same as that in the respective control young It was, however, found that analgesia declined faster in the morphine treated young than in the control young in the 20 and 26 days groups (the degree of analgesia was significantly lower at 90 min in the morphine treated young), whereas this was not the case in the 32

42 days old rats This was taken as a sign of residual tolerance to morphine Residual tolerance (significantly lower analgesic effect of morphine at 90 and 120 min) was also observed in the mothers 42 days after delivery, following treatment with morphine during pregnancy The young of the morphine treated mothers weighed significantly less at the age of 20 and 26 days than the control young This difference however, was not seen after 4 weeks of age

Key words Morphine – analgesia – sensitivity – young rats – adult rats – brain levels – tolerance

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A particularly high degree of analgesia has been reported after small doses of subcutaneous morphine in 12–13 days old rats At the age of 20–21 days higher doses of morphine were required in order to obtain the same degree of analgesia These doses were, however, far lower than those required to produce a degree of analgesia of a similar order of magnitude in experiments on adult female rats (JOHANNESSON & BECKER 1972) The results of this previous study moreover indicated that sensitivity to morphine analgesia in the young animals could vary with the body weight within the same age group In the present investigation we have therefore attempted a more systematical study of the sensitivity of rats at various ages to morphine analgesia, by using animals of as uniform a body weight as possible within the different age groups Included in the experiments were the young of rats treated with morphine during the pregnancy period as well as some of the mother rats This was done in order to study whether the development of morphine tolerance *in utero*, as demonstrated by JOHANNESSON & BECKER (1972), is of the same order as that which develops in the mothers

It appears to be an established experimental fact that the ratio between the amounts of morphine in the blood *versus* the brain in the rat increases with age (cf KUPFERBERG & WAY 1963, JOHANNESSON & WOODS 1964, JÓHANNESSON *et al* 1972), and MILTHERS (1959) contends that subcutaneous morphine is absorbed more efficiently in the young than in the adult rat These phenomena would obviously favour a greater sensitivity to morphine analgesia in young than in adult animals In the present investigation a subcutaneous dose of morphine was therefore administered to a number of animals in each age group and the amounts of morphine determined in the brain and plasma It should thus be possible to evaluate whether, and to what extent, the enhanced sensitivity to morphine analgesia in the young can be explained in terms of greater availability of the drug in plasma or to a greater access of the drug to the brain

Methods

Animals

Adult female Sprague Dawley (Simonsen) rats, around 230 g body weight, were housed in stainless steel cages and maintained under constant environmental conditions with free access to a commercial food preparation (Wayne-Lab-Blox, Allied Mills, Inc) and tap water The females were mated with male rats of known fertility from the same stock The finding of sperm in vaginal smears was taken as a sign of copulation, this time being designated as the first day of pregnancy The rats were kept in groups of three or four until day 21 or 22 of the pregnancy when they were separated and housed singly in cages furnished with pans covered with corn cob bedding Within 24 hours after delivery the young in each litter were counted and weighed and the litters standardized to eight pubs (4 males and 4 females) whenever possible The mothers and their young were taken off breeding pans 15 days after delivery and the young were weaned at the age of 26 days The young were used for the experiment at the age of 20 days (20–21 days) 26 days (26–27 days), 32 days (32–33 days) or at 42 days As far as possible, the litters were kept intact up to 26 days Experiments on 20 and 26 days old rats were therefore preferably carried out with whole litters (one or two at a time) After approximately 4 weeks of age the body weights of each of the animals in the litters were found to vary markedly, probably due to different growth rate of male and female young Experiments on 32 days and 42 days old rats were thus carried out on animals with as uniform a body weight as possible, taken from more than one litter, rather than from whole litters Care was taken, however to use an equal number of males and females in each group as far as was possible

The experiments also included three groups of adult female, non pregnant rats

The studies described here were conducted during the months of August, September and October 1972

Treatments

A stock solution containing 10 mg of morphine as the base per ml distilled water was prepared (10 ml each time) and all the solutions used for injections in the experiments on the degree of analgesia were fresh aqueous dilutions of this solution Injections were made subcutaneously in the middle of the back at the midline, using a short 25 gauge needle for the 20, 26 and 32 days old animals and 23 gauge needle for the 42 days old young and the adult animals and 1 ml tuberculin syringe graduated to deliver 0 01 ml Efforts were made to keep the volumes delivered from the syringe within a narrow range whatever the given doses and volumes per kg animal weight might be The average volume delivered from the syringe in all the analgesimetric experiments (except for maternal rats) was thus 0 43 ml ± 0 05 (S E M)

In order to study the effect of administration of morphine during pregnancy on the analgesic action of this substance in the resultant young, morphine (20 mg/kg) was injected subcutaneously (2 ml/kg) into a number of rats on days 17, 18, 19 and 20 of pregnancy (cf JÓHANNESSON & BECKER 1972) The young were then used for the experiment on the same days as the control young (young of maternal rats not injected with morphine)

The following doses of morphine (as the base) were used for analgesimetric experiments Figures in brackets denote the average body weight ± S E M of *all* animals used for analgesimetric experiments in the respective age groups

20 days old rats (40 7 g ± 0 74) 0 5, 1 0 or 2 0 mg/kg in a volume of 10 ml/kg,
26 days old rats (66 7 g ± 0 63) 1 0, 4 0 or 5 0 mg/kg in a volume of 6 ml/kg,
32 days old rats (103 0 g ± 1 94) 3 0, 4 0 or 7 0 mg/kg in a volume of 4 ml/kg,
42 days old rats (155 6 g ± 3 1) 10 0 or 15 0 mg/kg in a volume of 3 ml/kg,
Adult non pregnant, female rats (243 9 g ± 6 4) 5 0, 10 0 or 15 0 mg/kg in a volume of 2 ml/kg (These rats were taken from the same stock of female rats as those used for breeding)

The analgesic action of morphine was moreover measured in a number of maternal rats (both untreated and treated with morphine during pregnancy) on the 42nd day after delivery

The degree of analgesia was determined at four or five thirty minute intervals following a subcutaneous injection of morphine, using the hot plate method described by JÓHANNESSON & WOODS (1964), and expressed as the mean reaction time in seconds for a group of animals (i e the mean time in seconds which elapsed from the moment each animal was placed on the hot plate until it reacted to the heat) The animals were taken off the plate if they did not react in 30 seconds Thus 30 seconds is the highest score given In experiments on 20, 26 and 32 days old animals a restraining cylinder measuring 14×13 cm was used instead of the usual one used in experiments on adult animals

In order to study the distribution of morphine in brain and blood, a number of animals in each age group were injected subcutaneously with morphine 5 mg/kg (containing 5 or 10 % of radioactive morphine labelled with ^{14}C in the aminomethyl group) in the same volumes as stated above for animals (used for analgesic experiments) in the respective age groups Forty minutes later the animals were anaesthetized with ether While under ether anaesthesia, the animals were exsanguinated by heart puncture with heparinized syringes, the head cut off and the brain (including cerebellum and brain stem) was taken out The blood was centrifuged and the plasma collected Brain and plasma were stored frozen until analysed for morphine Brain and plasma from every two animals in the 20 and 26 days age groups were pooled and analysed collectively Radioactive morphine prepared by the method of ANDERSON & WOODS (1959), was obtained from Amersham/Searle The specific activity of the compound was 57 mci/mmol (150 µci/mg) and the radiochemical purity was found to be 98 % Non radioactive morphine was Morphine Sulfate, U S P

The estimation of radioactive morphine was essentially the same as that used by JÓHANNESSON & WOODS (1964) Levels of ^{14}C activity were determined in a Packard Model 3310 Tri Carb Liquid Scintillation Spectrometer equipped with automatic external standardization The quench correction and calculation of net disintegrations per minute from the counting rate were determined using a computer programme described by SPRATT & LAGE (1967) In experiments on the distribution of morphine the mixed drug (labelled and non labelled) was, as mentioned before, administered to the animals Appropriate multiplication factors were therefore used to calculate the total amounts of morphine in the brain and plasma The results (ng/g or ng/ml) refer to the total amounts of ' free or unconjugated drug only

Statistics

The t test was performed as described by GOLDSTEIN (1964) The level of significance was $P \leq 0 05$

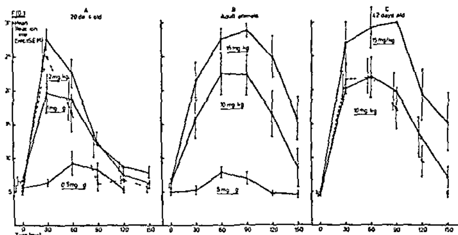


Fig 1 The analgesic action of morphine, expressed as the mean reaction time in seconds (± S E M) (ordinate) in 20 days old rats (A), adult female rats (B) and 42 days old rats (C) at 30, 60, 90, 120, and 150 min after subcutaneous injection of the drug (abscissa) The doses shown refer to the results of experiments depicted above Broken lines (– – – –) in parts A and C refer to experiments on the young of morphine treated mothers (given 2 mg/kg and 10 mg/kg, respectively) There were 8–16 animals in each group

Results

Lethality About one fourth of the maternal rats treated with morphine in the pregnancy period died within 24 hours after the first injection No deaths were observed in the control rats Litters of some maternal rats treated with morphine weighed significantly less at birth than those of the corresponding control rats Death rates of young in these litters were at times so high during the first two weeks that they did not fit into the experimental design (cf Methods) and were therefore removed from the experiments Other litters (the majority) of morphine treated mothers were of a similar or slightly less (not statistically significant) weight at birth as those of the control animals Death rates in these litters were similar to what was seen in litters of the corresponding control animals The young of these litters were subsequently used for analgesic experiments

Analgesimetry Subcutaneous morphine 0 5 mg/kg had only a slight analgesic action in the 20 days old rats If 1 mg/kg or 2 mg/kg was given, a high or very high degree of analgesia was obtained (fig 1A) The degree of analgesia rose sharply to a peak at 30 min and then quickly waned At maximum the analgesic action after 1 mg/kg and 2 mg/kg was thus about 20 sec and 28 sec., respectively, but had fallen to about 12 sec at 90 min

Much higher doses (7 5–10 times greater) were needed in order to obtain a comparable degree of analgesia in experiments on adult animals (fig 1B) However, the analgesic action neither increased nor decreased as sharply in the adult animals as in the 20 days old young and was therefore at a maximum at 60 or 90 min rather than at 30 minutes after the injection (figs. 1A and B) Statistical analysis showed that the degree of analgesia after 1 mg/kg morphine in the young and 10 mg/kg in the *adult animals did not differ* significantly at 30 or 60 min, while it differed significantly at 90 and 120 min In the groups which were given the highest doses (young 2 mg/kg, adult animals 15 mg/kg) the analgesic effect was significantly different at 30 min as well as at 90, 120 and 150 min after the injection At 60 min the difference was not significant.

The analgesic responses of the 42 days old rats were very similar to those of the adult animals (figs 1B and C) The rise and fall of analgesia was moreover similar in these two groups The degree of analgesia measured after the administration of same doses to rats in these groups was therefore not significantly different at any time interval

The results of experiments with 26 days and 32 days old rats are shown in figs 2A and B Morphine 1 mg/kg had a slight analgesic effect in 26 days old rats while a high degree of analgesia was obtained after a dose of 4 mg/kg and a very high effect after 5 mg/kg The intensity of analgesia, as well as its rise and decline, measured after the administration of morphine 4 mg/kg was very similar to that seen in the 20 days old rats after 1 mg/kg of morphine The analgesic responses measured after morphine 5 mg/kg in the 26 *days old rats were thus quite similar to those obtained in the 20 days old rats* after 2 mg/kg of morphine (figs 1A, 2A) Administration of 7 mg/kg of morphine to 32 days old rats gave a very high degree of analgesia which at 30 and 60 min was equal to that seen in adult animals after 15 mg/kg of morphine Unlike the 20 days and 26 days old rats, analgesia in the 32 days old rats was at a maximum at 60 rather than 30 min However, analgesia *declined significantly faster in 32 days old young than in the adult animals* (figs 1B and 2B) Analgesic responses measured in 32 days old rats after the administration of 4 mg/kg of morphine appeared to be lower than those measured in 26 days old rats given this dose (fig 2A and B) Statistical analysis however, showed the difference to be significant only at 90 min A dose of 3 mg/kg morphine was without analgesic effect in the 32 days old rats

A number of 20 days old young of morphine treated mothers were injected with 2 mg/kg morphine The results showed that the degree of analgesia was statistically the same at all time intervals as that measured in the young of the untreated mothers except at 90 min after the injection At this time interval the degree of analgesia was significantly lower in the young of

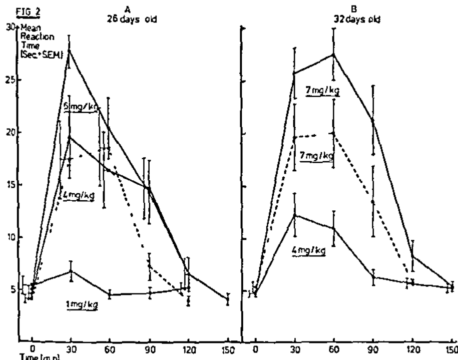


Fig 2. The analgesic action of morphine in 26 days and 32 days old rats
For explanation, see fig 1

the morphine treated mothers (fig 1A) Results of a similar experiment with 26 days old young of morphine treated mothers injected with 4 mg/kg are shown in fig 2A The degree of analgesia was significantly lower at 90 min than that measured in the control young At other time intervals observed the differences were not statistically significant In the 20 days group the average weight of the morphine young was 33 5 g ± 1 11 (S E M) as against 39 3 g ± 0 96 (S E M) of the control young This difference was statistically significant In the 26 days group the mean weight of the morphine treated young was also significantly lower (62 6 g ± 1 32 S E M) than that of the control young (67 1 g ± 0 77 S E M)

The degree of analgesia measured in 32 days old young of morphine treated mothers appeared to be appreciably lower than that in the control young (fig 2B) Statistical analysis showed, however, that at no time interval were the observed differences significant. The average weight of the young was also the same (control young 98 g, morphine young 96 g) At the age of 42 days, the analgesic action in young of the morphine treated mothers was at all time intervals statistically the same as that in the control young

Table 1

Amounts of morphine in brain (ng/g ± S E M) and plasma (ng/ml ± S E M) in 20 26, 32 and 42 days old rats and adult (female) rats 40 min after the subcutaneous injection of morphine 5 mg/kg Ratios between the amounts in plasma as against brain for each age group are included

N = number of animals in each group

	20 days old* N=14	26 days old* N=14	32 days old N=8	42 days old N=9	Adult animals N=8
Amounts in brain	504±14 5	242±8 1	213±8 6	203±6 4	202±3 5
Amounts in plasma	892±39 2	617±32.5	609±30 0	623±22.7	704±19 4
Ratios plasma/ brain	1 77	2 55	2 86	3 07	3 44

* Brains and plasma from two animals were pooled respectively and used for each analysis

(fig 1C) The average weight of these animals was also the same

Forty two days after delivery a group of 15 female rats were given 10 mg/kg of morphine The degree of analgesia measured was at all time intervals statistically the same as that seen in previous experiments on adult female rats (fig 1B) A group of 14 maternal rats which were treated with morphine during pregnancy were similarly used for experiment 42 days after delivery The analgesic effect of morphine was significantly lower at 90 and 120 min in these rats than in the control animals mentioned above At 30 and 60 min the analgesic effect was statistically the same in rats in both groups The average weight of the control animals was 302 g ± 0 57 (S E M) as against 290 g ± 7 5 (S E M) in the morphine treated animals This difference was not statistically significant

Distribution of morphine The results of these experiments are shown in table 1

Morphine was found in significantly greater amounts in the brain of the 20 days old rats than in the brain of rats in any other age group Brain levels of morphine were thus more than twofold lower in the 26 days old than in the 20 days old rats Somewhat lower levels, not statistically different from those in the 26 days old rats, were found in the brain of the 32 days old rats Morphine was in practically the same concentration in the brain of

42 days old and adult animals, the amounts being about 20 % lower than those in the brain of the 26 days old animals Statistical analysis showed that the above mentioned amounts in the brain of 42 days old and adult animals were significantly lower than those in the brain of the 26 days old animals Differences between morphine concentrations in the brain of the 42 days old and adult animals, on one side, and that of the 32 days old animals, on the other, were not statistically significant.

Morphine was also in significantly greater amounts in the plasma of the 20 days old animals than in the plasma of animals in any of the other age groups The plasma levels then fell by about one third and remained in the narrow range of about 610–620 ng/ml in the 26, 32 and 42 days old animals About 15 % higher amounts were found in the plasma of the adult animals These amounts were significantly greater than those mentioned above in the 26, 32 and 42 days old animals

The ratio between amounts of morphine in the plasma as against the brain increased steadily from the youngest age group to the adult animals In the adult animals it was thus approximately twice as great as in the 20 days old animals

Discussion

Sensitivity to morphine analgesia was very great in the youngest age group (20 days old) Sensitivity then waned rapidly during a six day period Two- to fourfold greater doses were thus needed in order to produce a similar degree of analgesia in 26 days old rats as in the younger animals (figs 1A and 2A) During the next six day period (26 to 32 days), sensitivity appeared to decline relatively less than during the previous period (fig 2B) Thus the degree of analgesia measured at 30 and 60 min after 4 mg/kg of morphine in 32 days old rats did not differ significantly from that obtained in 26 days old rats when the same dose was given However, a dose of 3 mg/kg of morphine was without any analgesic effect in the 32 days old rats (cf Results) The sensitivity then declined by a factor of two, at least, and was in the 42 days old animals practically at the adult levels (figs 1B and C) In conclusion, the results of analgesimetric experiments indicate that sensitivity to morphine analgesia declines by a factor of seven to ten from 20 days old as compared to adult animals, the level of sensitivity in the 32 days old animals is probably midway (figs 1 and 2 and Results)

The sharp decline in analgesic sensitivity from 20 to 26 days old animals was in our experiments matched by a significant decrease in the amounts of morphine determined in brain and plasma of the animals (table 1) Moreover, the ratio between the amounts of morphine in the plasma as against

the brain increased from 1 8 in the 20 days old animals to 2 6 at 26 days, thus indicating a lower permeability of the brain to morphine In other words, a rapid development of the so-called brain blood barrier to morphine, which is virtually absent at birth (JOHANNESSON *et al* 1972), must have occurred during this period of time A decreased permeability of the brain in the 26 days old animals, as compared to the 20 days old animals, must therefore have some bearing on the decreased sensitivity to morphine in these animals This is in agreement with the results of KUPFERBERG & WAY (1963) *from* experiments on morphine lethality in young rats It thus seems likely that the decrease in sensitivity to morphine analgesia and lethality which occurs in rats from about two weeks to about four weeks of age, is to a large extent determined by the development of a brain blood barrier to morphine More over we believe that the developmental stage of this barrier can vary with the body weight of the animals within this interval This is borne out by the fact that in a previous study (JOHANNESSON *et al* 1972) we found after sub cutaneous morphine no statistically significant difference between the amounts in the brain and blood of 20 days old young taken from unstandardized litters (body weight about 30 g) which is in sharp contrast to the results of experiments on 20 days old young in the present study with an average body weight of about 40 g. Such difference in the developmental stage of the brain blood barrier would furthermore explain why sensitivity to morphine analgesia in 20 days old rats may vary with the body weight of the animals (cf JOHANNESSON & BECKER 1972)

It is mentioned above that the amounts of morphine in the plasma were significantly lower in 26 days old than in 20 days old rats (table 1) A possible explanation for this phenomenon could be a slower rate of conjugation of morphine in the younger age group during the initial period after injection (cf KUPFERBERG & WAY 1963) Another possible explanation is the so called self depression in the subcutaneous absorption of morphine due to the release of histamine and serotonin (for review, see SCHOU 1961) which is much less efficient or absent in young rats than in adult animals (MILTHERS 1959) However the validity of this hypothesis has subsequently been questioned by KUPFERBERG & WAY (1963) Whatever the explanation it is obvious that the availability of morphine in plasma was greater in 20 days old than in 26 days old rats This fact must therefore, in the presence of a relatively deficient brain blood barrier to morphine at this and younger age levels, be of importance with regard to the high concen tration of morphine in the brain of the 20 days old rats A high degree of availability of morphine in the plasma could also be a contributory cause of the exceptionally great sensitivity to morphine analgesia in 12–13 days old rats, an age level at which the brain blood barrier to morphine is probably nonexistent (cf JOHANNESSON & BECKER 1972, JÓHANNESSON *et al*

1972) In these studies it was shown that 0 45 mg/kg subcutaneously of morphine gave a high degree of analgesia in rats of this age or comparable to that which was obtained after 2 mg/kg of morphine in 20 days old rats in the present investigation (fig. 1A) However, the results of our experiments show clearly that an increase in availability of morphine in the plasma does not necessarily result in greater amounts in the brain Thus, even though the levels of morphine were significantly higher in the plasma of adult animals than in the 26 days old animals, morphine was actually present in significantly lower amounts in the brain of the adult than in the brain of the younger animals Thus in our experiments there is no ready explanation for the relatively high amounts of morphine in the plasma of the adult animals

Although morphine was present in somewhat lower amounts in the brain of 32 days old than in 26 days old rats, the difference was not statistically significant Morphine, on the other hand, was found in significantly lower amounts in the brain of 42 days old and adult animals than in the brain of the 26 days old animals (table 1 and Results) The lower sensitivity to morphine analgesia in 42 days old and adult animals is thus matched, to some extent at any rate, by decreased amounts of morphine in the brain Morphine was, however, found in the same amounts in the brain of 32, 42 days old and adult animals (table 1) It should be recalled here that results of the analgesimetric experiments indicate that sensitivity to morphine analgesia is appreciably greater in 32 days old rats than in 42 days old or the adult animals (figs. 1B and C and fig 2B) It is thus possible that young animals have to some extent a greater sensitivity *per se* to morphine analgesia than adult animals In summary therefore, the greater sensitivity to morphine analgesia in young rats seems to be determined by 1) deficiency in the brain blood barrier to morphine, 2) increased availability of morphine in the plasma in the presence of a deficient brain blood barrier (this applies especially to the youngest animals), and 3) a possibly greater intrinsic sensitivity to morphine analgesia

As mentioned above there is a quicker rise and decline of analgesia in the two youngest age groups (and in part in the 32 days old rats) than in the adult or 42 days old animals (figs 1 and 2 and Results) A possible explanation for this phenomenon could be that the brain and blood levels of morphine equilibrate faster in young animals, where the brain blood barrier is relatively little developed, than in older animals with a more developed barrier If this is so, then the amounts of morphine in the brain of the young should rise and recede relatively quickly with rising and falling levels in the plasma In the adult animals, on the other hand, a longer lag period would be observed for movements in either direction Results of experiments with two barbituric acid derivatives, where equilibrium

amounts in brain and plasma occur at a different rate, support this assumption (GOLDSTEIN & ARONOW 1960) However, KUPFERBERG & WAY (1963) have found that brain levels of morphine fall at a slower rate in 16 days than in 32 days old rats If this holds true, a different relationship must exist between the amounts of morphine in the brain and the degree of analgesia in young and in adult rats

If morphine is administered to maternal rats late in pregnancy, tolerance to morphine analgesia results This was demonstrated in the young at the age of 12–13 days and in the mothers at four weeks after delivery (JÓHANNESSON & BECKER 1972) In the present investigation, young of morphine treated mothers in all age groups showed statistically the same degree of analgesia at its maximum as their controls (young of mothers not treated with morphine) However, the degree of analgesia declined significantly faster in the young of morphine treated mothers at the age of 20 and 26 days than in their controls The same pattern was seen in experiments with maternal rats at 42 days after delivery The same trend, although not reaching the level of significance, was seen in 32 days old young of morphine treated mothers, while at the age of 42 days, it had almost disappeared (cf figs 1 and 2 and Results) In this connection the results of JOHANNESSON & WOODS (1964) should be recalled They performed experiments on highly tolerant rats, (pre treated with high doses of morphine or codeine) and showed that tolerance to morphine or codeine analgesia results in a lesser degree or intensity of analgesia as well as in shorter duration of action We therefore believe that the shortened duration of analgesia in the above mentioned groups of animals denotes a kind of tolerance which would also be evident at an earlier stage as a lower intensity of analgesia The results moreover indicate that tolerance to morphine analgesia that develops *in utero* is of a shorter duration than that which develops in the maternal rats

Although offspring of morphine treated mothers may have the same or similar weight at birth as the young of untreated mothers, they gain weight at a slower rate than the control young (JOHANNESSON & BECKER 1972) This difference seems to disappear at about 4 weeks of age (cf Results) In this connection it is remarkable that the vestigial signs of tolerance developed *in utero*, and which are mentioned above, disappear at about the same age The significance of these findings remains to be elucidated

The lethal effect of morphine in maternal rats was greater in these experiments than in a previous study (JOHANNESSON & BECKER 1972) The effect on the young was moreover on occasions greater in the present investigation than in the previous one In the case of high lethality in litters of morphine treated mothers, the surviving young were, however as a rule not used (cf Results) Results of experiments with the young of morphine treated mothers should therefore be comparable in the two studies

Acknowledgements

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Material and Methods

Animals

Four groups of male Sprague Dawley rats, weighing 180–200 g were used The animals were kept on a standard diet Group I (= control group) received tap water, group II 15 % (v/v) ethanol, group III 0 05 % (w/v) phenobarbital and group IV both 15 % (v/v) ethanol and 0 05 % (w/v) phenobarbital in drinking water *ad libitum*

Preparation of livers

The animals were killed by decapitation at the same time of day, between 7 00 and 8 00 a.m, and the livers were immediately excised and rinsed with ice-cold 0 1 M phosphate buffer at pH 7 4 All subsequent tissue manipulations were conducted at 0–4° The livers were homogenized in 4 volumes of cold 0 1 M phosphate buffer at pH 7 4 in a Potter Elvehjelm homogenizer The homogenates were centrifuged at 12 000 × g at 4° for 20 minutes in a refrigerated MSE superspeed 50 ultracentrifuge The 12,000 × g supernatant was used as the enzyme preparation when assaying methyl aniline N-demethylase and *p* nitrobenzoic acid reductase activities Some of the 12 000 × g supernatant fraction was centrifuged at 105 000 × g at 4° for one hour and the pellet formed was suspended in the phosphate buffer in such a way that 1 ml suspension corresponded to 800 mg liver This suspension of 105 000 × g pellet was used in assaying the cytochrome P 450 and microsomal protein contents

Enzyme assays

For the determination of methylaniline N-demethylase activity the incubation mixture contained 2 ml enzyme preparation, 120 µmol nicotinamide, 10 µmol $MgCl_2$, 200 µmol KCl, 6 µmol glucose-6 phosphate, 0 25 µmol NADP, and sufficient phosphate buffer (0 1 M, pH 7 4) to make a final volume of 4 ml 5 µmol methylaniline was used as substrate and the incubation for 30 minutes took place at 37° in a metabolic shaker under the atmosphere of air In the assay of *p* nitrobenzoic acid reductase activity the conditions were similar except that nicotinamide was omitted because it has been found to inhibit the reaction (Vorne & Arvela 1971), so that an atmosphere of nitrogen was used instead of air, and 3 µmol *p*-nitrobenzoic acid was used as the substrate and the incubation time was 60 minutes

The rate of methylaniline N demethylation was assayed by measuring the aniline formed during incubation (Brodie & Axelrod 1948) *p* Nitrobenzoic acid reduction was assayed by measuring the *p* aminobenzoic acid formed (Fouts & Brodie 1957) The cytochrome P-450 content was measured according to the method described by Omura & Sato (1964), by using a Unicam 800 spectrophotometer with an external recorder Microsomal protein was measured by a biuret procedure (Laune 1957)

Results

Effect of ethanol and/or phenobarbital on the rate of metabolism of methylaniline and p nitrobenzoic acid

Values for methylaniline demethylation are given in table 1 and those for *p* nitrobenzoic acid reduction in table 2 An increase in the N demethy' activity was observed in the ethanol group The maximum increase (was measured after treatment for 3 days In the phenobarbital g

Table 1

Changes with time in the N demethylation of methylaniline during treatment with ethanol (15 %, v/v) and/or phenobarbital (0 05 %, w/v) in drinking water

Duration of treatment (days)	Aniline formed µmol/g liver/60 min Mean±S E M Control	Ethanol	Phenobarbital	Ethanol + phenobarbital
1	1 3±0 2 (4)	1 6±0 1 (4)	1 6±0 3 (4)	1 4±0 3 (4)
3	1 6±0 2 (5)	3 0±0 5 (5) *	3 9±1 0 (5) *	4 8±1 2 (6) *
7	1 4±0.2 (5)	2 5±0 2 (5) **	3 9±0 4 (5) ***	4 8±0 7 (5) **
14	1 3±0 8 (4)	1 8±0 2 (4) *	4 7±1 0 (4) ***	5 5±1 3 (4) **

The numerals in brackets indicate the numbers of animals.
Significance of differences from control *=P < 0 05, **=P < 0 01, ***=P < 0 001

increase of the N demethylase activity continued throughout the experiment and after 14 days treatment, N demethylation was 262 % greater than in the controls Combined ethanol and phenobarbital treatment increased the N demethylation activity by 323 %

The rate of reduction of *p* nitrobenzoic acid was decreased in the ethanol group (table 2) A maximum inhibition (47 %) was observed after ethanol ingestion for 3 days In the phenobarbital treated group the nitroreductase activity increased and this increase continued throughout the experiment, after 14 days the enzyme activity was 277 % higher When the animals received ethanol and phenobarbital together, the nitroreductase activity was increased by 64 % after 7 days, and by 137 % after 14 days Thus ethanol inhibited the stimulating effect of phenobarbital on the nitroreductase, after 3 days treatment the inhibition was 83 % and after 14 days 49 %

Table 2

Changes with time in the reduction of *p* nitrobenzoic acid during treatment with ethanol (15 % v/v) and/or phenobarbital (0 05 % w/v) in drinking water

Duration of treatment (days)	*p* Aminobenzoic acid formed µmol/g liver/60 min Mean±S E M Control	Ethanol	Phenobarbital	Ethanol + phenobarbital
1	2 9±0 1 (4)	2 9±0 1 (4)	2.8±0 1 (4)	2 7±0 1 (4)
3	3 2±0 3 (5)	1 7±0 1 (5) *	6 7±0 5 (5) *	3 8±0 7 (5)
7	3 9±0 1 (5)	2.1±0.2 (5) **	9 5±0 7 (5) ***	6 4±0 5 (5) **
14	3 5±0 3 (4)	2 2±0 2 (4) *	13 2±1 6 (4) ***	8 3±0 9 (4) **

The numerals in brackets indicate the number of animals
Significance of differences from control *=P < 0 05 **=P < 0 01 ***=P < 0 001

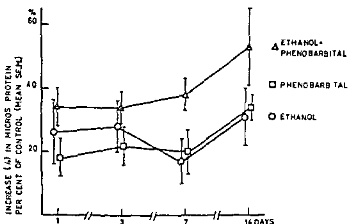


Fig. 1 Increase in microsomal protein during treatment with ethanol (15 %, v/v) and/or phenobarbital (0 05 %, w/v) in drinking water Each point represents the mean value of 4–6 animals

Changes in the concentration of microsomal protein and cytochrome P-450

An increase in the microsomal protein content by about 30 % was noted in the ethanol group, which was approximately equal to the increase measured after the ingestion of phenobarbital alone (fig 1) The combined

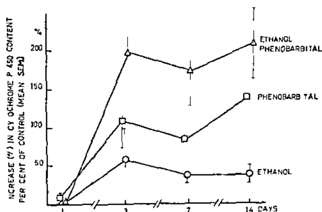


Fig. 2 Increase in cytochrome P-450 content during treatment with ethanol (15 %, v/v) and/or phenobarbital (0 05 %, w/v) in drinking water The cytochrome content was calculated as μmol/g liver Each point represents the mean value of

treatment with ethanol and phenobarbital brought about an increase in the microsomal protein content which was significantly greater than that noted after the ingestion of ethanol alone Treatment with ethanol, like that with phenobarbital, increased the cytochrome P-450 concentration (fig 2) The increase was highest in the ethanol phenobarbital group and least in the ethanol group

Discussion

The increase in the rate of N demethylation of methylaniline caused by ethanol and phenobarbital together was approximately equal to the sum of the respective increases by each agent The changes in the cytochrome P 450 and microsomal protein content caused by ethanol and phenobarbital alone and together were similar in tendency to the changes in N demethylase activity The effects of ethanol on the N-demethylase activity, cytochrome P-450 and microsomal protein are presumably associated with the increase in the smooth endoplasmic reticulum (ISERI et al 1966) as also occurs with phenobarbital and other inducers (CONNEY 1967) Phenobarbital increased the N demethylase activity throughout the experiment, whereas the effect of ethanol on this was most pronounced on the third day and thereafter decreased This suggests a different mechanism of action of these agents, and could also explain their additive effects The changes in the enzyme activities studied caused by ethanol could result from a changed caloric intake, as a substantial part of the caloric intake was derived from ethanol It is known that changes in diet can affect the microsomal enzyme activity (KATO 1967, KHANNA *et al* 1972)

Nitroreductase activity is present in the soluble fraction as well as in microsomes (KATO *et al* 1969) In our work nitroreductase was assayed in the 12 000 × g supernatant fraction, and thus both microsomal enzyme activity and the enzyme activity of the soluble fraction were measured KATO *et al* (1969) noted a marked increase in microsomal nitroreductase activity but no significant change in that of the soluble fraction in connection with phenobarbital induction In our work ethanol inhibited nitroreductase and also its induction by phenobarbital induction Its inhibitory effect was most pronounced when given together with phenobarbital, which also suggests that ethanol particularly inhibits the microsomal reduction of *p* nitrobenzoic acid Several nitroreductases have been shown to be responsible for microsomal nitroreduction (SASAME & GILLETTE 1969) Ethanol did not completely inhibit the induction of nitroreduction by phenobarbital, which may be due to the fact that ethanol inhibits only some of the nitroreductases

ARIAS *et al.* (1969) have noted that the rates of synthesis of individual proteins associated with the endoplasmic reticulum can vary independently, either increasing or decreasing, and this may explain the different actions of ethanol and phenobarbital on the enzyme activities studied

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Manganese Induced Histochemical and Histological Alterations in Gastrointestinal Mucosa of Guinea Pigs

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Abstract Manganese chloride (10 mg/kg) was administered orally to guinea pigs for a period of 30 days The histochemical and histological alterations in gastric mucosa consisted of loss of mucin and pepsinogen granules adenosine triphos phatase and glucose-6 phosphatase activities and patchy necrosis of the epi thelium The intestinal villi also showed patchy necrosis, the activities of adenosine triphosphatase and glucose-6 phosphatase were diminished while there was an increase in the activity of acid phosphatase in the mucosa. Excess of manganese in the gastrointestinal tract produces functional and structural alter ations in the mucosal cells The exact mechanism of action needs further study

Key words Manganese toxicity – gastrointestinal tract

Manganese is known to produce neurological disorders when inhaled by miners and the lung has long been thought as the main route of entry of this metal (COUPER 1837 RODIER 1955, TANAKA & LIEBEN 1969) Recently it has been shown that the inhaled manganese finds its way into the gastro-intestinal tract from which it is either absorbed or eliminated (MENA *et al* 1969) Irrespective of the mode of entry the importance of the gastro-intestinal tract in manganese toxicity assumes great significance

Administration of large doses of manganese salt into the stomach of animals produces corrosion of the gastric walls and the intestines (VON OETTINGEN 1935) In view of the paucity of the literature and the importance of the gastrointestinal tract in manganese toxicity, histological and histo-chemical studies of the gastrointestinal mucosa were undertaken in guinea pigs after oral administration of manganese chloride

Materials and methods

Seventy male guinea pigs of average weight 350 g from I T R C colony were divided into three groups Group I consisting of 20 animals were kept as normal controls Group II consisting of 20 animals were given orally with a cannula 1 ml physiological saline daily for a period of 30 days and group III consisting of 30 animals were given orally manganese chloride (10 mg/kg) in a similar manner daily for the same period

Two animals from group II and six animals from group III died during the course of the experiment The dead animals were autopsied but the cause of death could not be ascertained The remaining animals were sacrificed at the end of the experiment by ether anaesthesia The animals were chilled on crushed ice and the stomach and intestinal segments were taken out and rinsed with ice cold solution of 5 % sucrose The stomach was cut open along the greater curvature and small pieces from different parts were cut out Approximately 1 cm long segments were cut from the jejunum ileum and colon These tissues were treated in three different ways (a) Small pieces were fixed in chilled 1 % calcium formol solution for 24 hrs at 4°, rinsed with 5 % sucrose blotted gently and put in gum sucrose for 24 hrs at 4°, after which free floating sections 10–15 μ thick were cut in a freezing microtome (b) Unfixed segments were frozen in liquid nitrogen and directly mounted on to microtome chucks and kept at −20° until sections 5–6 μ thick were cut in cryostat the sections were then mounted on cover slips for enzymic studies (c) Small pieces of stomach were fixed in Regaud s fixative (McManus & Mowry 1965)

The remaining stomach specimens were placed on stiff paper and together with the remaining segments of intestines were fixed in neutral buffered formalin The tissues were dehydrated in graded alcohol, cleared in toluene and embedded in paraffin Sections were then cut and stained with haematoxylin and eosin PAS alcian blue (McManus & Mowry 1965) and for pepsinogen granules by Bensley s neutral gentian stain (Cowdry 1948) Other histochemical methods included the demonstration of alkaline and acid phosphatases (Gomori 1939 & 1941) adenosine triphosphatase (ATPase) by the method of Padykula & Herman (1955) and glucose 6 phosphatase by the method of Wachstein & Meisel (1956) For comparison sections of the same region of the gastrointestinal tract of control animals were studied for enzymic activity Controls were also studied for each enzyme by incubation in the respective incubation media without specific substrate and at the same time one cryostat section from each block was stained with haematoxylin and eosin

Results

Macroscopic No gross abnormality was observed on the serosal surface of the stomach in all the groups After cutting open along the greater curvature, the mucosa was found to be greyish pink in colour with numerous folds in groups I and II A few small pin point haemorrhages and abrasions were noticed along the greater curvature of the stomach in group III The intestines did not show any gross abnormality

Microscopic The gastric and intestinal architecture in groups I and II was normal and almost resembled the histology of the gastrointestinal tract described by Bloom & Fawcett (1966)



Fig 1 Gastric mucosa of guinea pig after oral administration of manganese for thirty days showing necrosis of mucosal glands interglandular oedema and congestion Haematoxylin and eosin Magnification ×300

The chief cells which were present in the lower half of the gastric mucosa were filled with pepsinogen granules and the mucous cells were distended with mucin

Stomach In group III patchy morphological changes were observed throughout the mucosa The surface epithelial cells were ragged with a variable intensity of nuclear staining and irregular positioning of the nucleus within the cells, in some places the superficial epithelium was totally absent The glands were atrophic and distorted with reduced number of cells Some of the cells were lying free and the nests of glandular cells were no longer attached together in an orderly arrangement In some places, degenerated mucosal cells with pyknotic nuclei and homogenous eosinophilic cytoplasm were seen There was oedema of the interglandular tissue with congested blood vessels and fine connective tissue Red blood cells were seen lying free in the interglandular spaces The lamina propria showed dilated and congested blood vessels, with infiltration of round cells (fig. 1) The pepsinogen granules were absent from most of the chief cells (figs 2 and 3) and mucous cells also showed marked depletion of mucin granules as was evident from their histochemical reactions

Adenosine triphosphatase In the animals of groups I and II marked activity of ATPase was observed in the supranuclear and along the lateral cell membrane of the superficial epithelial cells Mild activity was seen in the glandular cells in both the fundus and pyloric regions. In group III

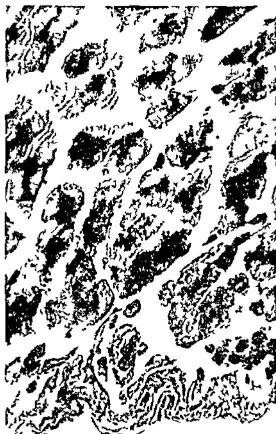


Fig 2. Gastric mucosa of control guinea pig showing pepsinogen granules in the chief cells Bensley s neutral gentian stain Magnification ×680

very faint activity of ATPase was observed in the supranuclear and along the lateral cell membrane of the glandular cells In some places the activity was totally absent

Glucose 6 phosphatase In groups I and II marked activity of glucose 6 phosphatase was observed in the gastric mucosal cells The reaction was diffuse throughout the cell cytoplasm In manganese fed animals the activity of this enzyme was very faint in the cytoplasm of the mucosal cells, and in some places activity was totally absent

Acid phosphatase activity was not demonstrated in the cells of the gastric mucosa There was no change in the activity of alkaline phosphatase in the manganese treated animals

Small intestines In the animals of group III the mucosa of the jejunum and ileum showed flattening of villi In some places villi were absent, the surface epithelium was denuded, the nuclei were pyknotic and there was a



Fig. 3 Gastric mucosa of guinea pig after oral administrat on of manganese for thirty days peps nogen granules are absent in the chief cells Bensley s neutral gent an stain Magn f cat on ×680

collection of inflammatory cells The lamina propria contained inflammatory cells namely lymphocytes plasma cells and histiocytes (fig. 4) The glandular epithelium was very much narrowed and there was focal involvement of glandular epithelial cells showing degeneration and necrosis

Acid phosphatase Acid phosphatase activity in the group I and II animals was located in the cytoplasm of the superficial epithelial cells and in the histiocytes in the lamina propria in the mucosa of jejunum and ileum In the animals of group III the acid phosphatase activity was seen in the cytoplasm of surface epithelial cells The histiocytes in the lamina propria showed a very intense reaction for this enzyme (figs 5 and 6) No significant alteration in the activity of alkaline phosphatase was observed in the epithelial cells of villi in all the groups

Adenosine triphosphatase In the animals of groups I and II the brush



Fig 4 Jejunal epithelium of guinea pig showing flattened and necrosed villi with inflammatory reaction Haematoxylin and eosin Magnification ×300

border and deeper glands showed intense activity of ATPase In the mucosa of the group III animals, patchy loss of activity of ATPase was observed in the brush border and deeper glandular cells, while in some places there was no activity



Fig 5 Jejunal villi of control guinea pig showing acid phosphatase activity in the supra nuclear region of the cells and in the histiocytes in the lamina propria Magnification ×600

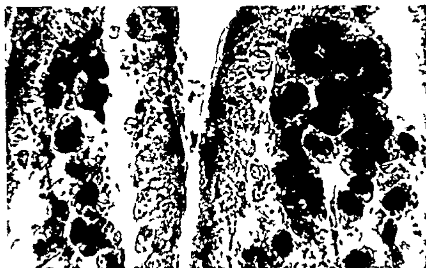


Fig 6 Acid phosphatase activity in jejunal villi of guinea pig after oral administration of manganese for thirty days the localisation of enzyme activity is similar in the surface epithelial cells but the number of acid phosphatase containing histiocytes in the lamina propria is very much increased. Magnification ×600

Glucose 6 phosphatase In the animals of groups I and II, superficial mucosal cells, cells covering the sides of villi, crypts and deep glandular cells reacted intensely for glucose 6 phosphatase activity There was uniform

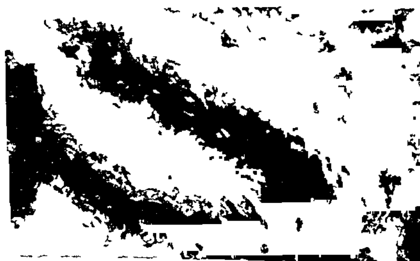


Fig 7 Glucose-6-phosphatase activity in the mucosa of control guinea pig ileum showing intense reaction in the mucosal cells Magnification × 375

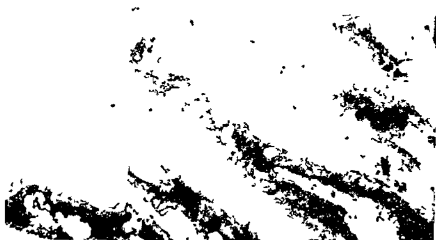


Fig 8 Glucose-6 phosphatase activity in the mucosa of guinea pig ileum, after oral administration of manganese for thirty days, showing very faint reaction Magnification ×375

reaction in the cytoplasm In the mucosa of the group III animals a very faint reaction of glucose 6 phosphatase was seen in the superficial cells, cells covering the sides of villi, crypts and deep glandular cells In some places no activity was observed (figs 7 & 8)

Large intestines No pathomorphological alterations were observed in the large intestines

Discussion

Histological changes in the gastrointestinal tract are known to occur with chemicals like salicylates, phenylbutazone and salts of copper and cobalt (PLANTEYDT & WILLIGHAGEN 1960, ZAIDI & MUKERJI 1962, BROWNING 1969, CHANDRA & SINGH 1967) In our experiments oral administration of manganese chloride in guinea pigs for thirty days has resulted in patchy necrosis of the mucosa of the stomach and small intestines Necrosis of the bronchial mucosa after intratracheal administration of manganese chloride in rats was observed by DAVIES & HARDING (1949) The histochemical alterations showed a marked decrease in mucin and pepsinogen granules in the gastric mucosa and in the activities of adenosine triphosphatase and glucose-6 phosphatase in the mucosa of the stomach and small intestines

The disturbed cellular functions as evident from histochemical changes are not in proportion to the histological evidence of cellular damage in the mucosa Similar disproportion in the functional state and histological changes in gastritis have been observed by ROHNER & WELSH (1967) Manganese may produce structural changes not appreciable by light microscopy, thus inhibiting the cellular functions or in the first instance it may alter the cellular functions rather than produce actual destruction

The significance of the intracellular mucosal enzymes in gastrointestinal physiology is poorly understood The presence of ATPase in the gastric and intestinal mucosa is probably concerned in the release of energy required for the absorption, transport and synthetic activity of the cell Glucose 6 phosphatase helps in the metabolism of carbohydrate and finally in the absorption of glucose in the blood stream Thus the loss of ATPase and glucose 6-phosphatase activity in the gastrointestinal mucosa in our experiments indicates impairment of metabolic activity of the cell under the toxic influence of manganese A decreased activity of ATPase in brain due to manganese toxicity has been reported previously (CHANDRA 1972) Inhibition of ATPase activity also occurs in the nephrons of experimental animals after the intravenous administration of manganese chloride (JONEK *et al* 1965)

The activity of acid phosphatase was not demonstrable in the stomach The absence of acid phosphatase activity in the mucosa of the stomach of dogs, rats and cats was also reported by GOMORI in 1941 (loc cit) The increase in the activity of acid phosphatase in the histiocytes of the lamina propria in manganese treated animals may be due to an increased histiocytic reaction resulting from an excess of manganese An increase in acid phosphatase activity has been observed in the kidneys and cerebellum of rabbits intoxicated with manganese (JONEK *et al* 1965, *loc cit*, JONEK *et al* 1966) The activity of alkaline phosphatase was not affected by manganese as has been observed in our previous studies on the brain (CHANDRA 1972 *loc cit*) The present investigations have shown that the excess of manganese in the gastrointestinal tract produces functional and structural alterations in the mucosal cells The exact mechanism of action remains to be elucidated

Acknowledgement

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Effects of Insulin on Glucose Metabolism in Vascular and Intestinal Smooth Muscle

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Abstract The effect of insulin (0 1 U/ml) on glucose metabolism was studied in bovine mesenteric arteries and rabbit colon smooth muscle In both tissues the glucose uptake was enhanced by insulin after an incubation period of 180 min and at a glucose concentration of 5 6 mM Insulin also augmented the incorporation of glucose ^{14}C into glycogen as well as the glycogen content and the lactate elimination Glycogen synthetase I activity was increased by insulin The total glycogen synthetase phosphorylase *a* or total phosphorylase activities were not affected The effects of insulin on the glucose metabolism in bovine mesenteric arteries and rabbit colon smooth muscle were qualitatively similar to those in rat skeletal muscle but the effects were less pronounced and appeared later

Key words Vascular smooth muscle – intestinal smooth muscle – insulin – glucose metabolism

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There are few reports on the effect of insulin on the glucose metabolism in arterial tissue In the rabbit aorta a small but consistent increase in the glycogen content and in the incorporation of glucose carbon into glycogen and lipids was reported by MULCAHY & WINEGRAD (1962) They found no effect of insulin on the glucose uptake, lactate metabolism or the oxidation of glucose to CO_2 In bovine mesenteric arteries insulin increased the glycogen content (LUNDHOLM & MOHME LUNDHOLM 1963) In the rat aorta WERTHEIMER & BEN-TOR (1962) observed a stimulation of the glucose uptake by insulin. URRUTIA *et al* (1962), however, found no effect of insulin on the glucose oxidation or the incorporation of glucose carbon into glycogen and lipids in a rat aorta preparation which had been carefully freed from perivascular adipose tissue

It was found earlier (ARNQVIST 1971, 1972) that the tissue accumulation of glucose carbon from ^{14}C-labelled glucose was moderately increased by insulin in the rat aorta, bovine mesenteric arteries and rabbit colon smooth

muscle The membrane transport of monosaccharide (3-O-methylglucose) in bovine mesenteric arteries and rabbit colon smooth muscle had the characteristics of facilitated diffusion and was stimulated by insulin (ARNQVIST 1972)

The aim of the present study was to further investigate the effect of insulin on the glucose metabolism in vascular smooth muscle Bovine mesenteric arteries, which have a high content of smooth muscle (DUCRET 1930), were used Parallel experiments were done on an almost pure smooth muscle preparation – the muscle layer of the rabbit colon The effect of insulin on glucose uptake, glycogen content, incorporation of glucose-^{14}C into glycogen and lactate elimination was measured The influence of insulin on the key enzymes in glycogen metabolism, glycogen synthetase and phosphorylase, was also determined

Material and Methods

Experimental procedure Bovine mesenteric arteries were removed from full grown cattle about 30 min after slaughter and transported to the laboratory in Krebs Henseleit bicarbonate buffer at 37° Pieces of intima media weighing 50–100 mg were dissected from the arteries as previously described (ARNQVIST 1972) Rabbit colon smooth muscle was obtained from rabbits weighing 2–3 kg which were starved for 20–24 hrs before the experiments The rabbits were killed by a blow on the neck and pieces of rabbit colon smooth muscle weighing 30–60 mg were prepared according to ARNQVIST (1971) Fed male rats (60–70 g) of the Sprague Dawley strain were killed by cervical fracture and the intact rat hemidiaphragms were used as described by KONO & COLOWICK (1961)

Incubations were performed in Krebs Henseleit bicarbonate buffer with the following composition (mM) 120 NaCl, 4 7 KCl 1 3 $CaCl_2$ 1 2 $MgSO_4$, 1 2 KH_2PO_4 25 $NaHCO_3$ The buffer was equilibrated with a mixture of 95 % O_2 and 5 % CO_2 at 37° which gave pH 7 4 1 mg glucose/ml was added to the preincubation medium Tissue samples were incubated in 4 ml medium, except for the intact rat hemidiaphragms which were incubated in 8 ml medium For glucose uptake determinations tissue samples were incubated in 1 8 or 2 3 ml medium

Analytical methods The methods used for the determination of glucose uptake, glycogen content and lactate have been described in detail in a previous report (ARNQVIST 1973) In brief glucose was determined by hexokinase, glucose 6 phosphate dehydrogenase (SLEIN 1962), glycogen with an amylo-glucosidase method (KEPPLER & DECKLER 1970) and lactate with lactate dehydrogenase (LUNDHOLM *et al* 1963)

For the determination of the incorporation of glucose ^{14}C into glycogen, 50–100 mg of tissue was digested in 1 ml 1 M KOH, 10 mg of unlabelled glycogen was added and the glycogen was precipitated overnight at 4° in 70 % ethanol and 60 mM Na_2SO_4 The precipitate was washed twice in 1 ml of 66 % ethanol, dried and dissolved in 0 5 ml of water After addition of scintillation fluid (Instagel) the samples were counted for radioactivity

For measurement of the glycogen synthetase activity the tissue was homogenized in Tris acetate 50 mM (pH 7 8) EDTA 2 mM and NaF 20 mM (1 20 w/v) Glycogen synthetase was assayed in a 1 60 dilution of the muscle extract by measuring the incorporation of UDPG ^{14}C into glycogen (VILLAR PAL 19 he presence

of glucose-6 phosphate (total glycogen synthetase activity) and in the absence of glucose-6-phosphate (independent form of glycogen synthetase or glycogen synthetase I) Tissue samples for phosphorylase assay were homogenized in citrate buffer 20 mM

in the absence of AMP (phosphorylase *a* activity)

All chemicals were of analytical grade UDPG ^{14}C and D glucose ^{14}C (U) were obtained from the Radiochemical Centre, Amersham, England Instagel was purchased from Packard Instr Co Inc Monocomponent pork insulin (lot No Mc S-970-AC) was a gift from Novo, Copenhagen

Statistical analysis Mean values are given ± S. E M Adjacent tissue samples from the same animal were used as test and control preparations The significance of the effect was calculated by Student's t test from the difference between these paired samples

Results

Effect of insulin on glucose uptake In rat skeletal muscle the glucose uptake was increased by insulin (0 1 U/ml) after an incubation period of 60 min (Chaudry & Gould 1969) In bovine mesenteric arteries and rabbit colon smooth muscle no effect of insulin (0 1 U/ml) could be demonstrated after incubation for 60 min in 5 6 mM glucose However, when determined after an incubation period of 180 min, the glucose uptake was moderately increased in both tissues as is shown in table 1 At a glucose concentration of 44 4 mM the basal glucose uptake in bovine mesenteric arteries was increased but insulin had no significant effect (table 1)

Table 1

Effect of insulin (0 1 U/ml) on glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle determined after an incubation period of 180 min Glucose uptake is calculated as μmol glucose/g tissue wet weight × 180 min The results are given as mean values ± S E M The numbers of observations are given in brackets

Preparation	Glucose concentration	Glucose uptake		
		Control	Insulin	Effect
Bovine mesenteric arteries	5 6	10 3±1 0	13 0±1 0	2 7±0 9 (13) P < 0 01
	44 4	20 4±5 1	21 5±4 6	1 1±4 2 (10) N S
Rabbit colon smooth muscle	5 6	31 5±3 2	35 9±3 2	4 4±1 1 (7) P

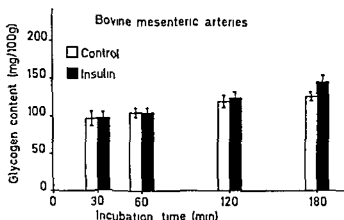


Fig 1a Effect of insulin (0 1 U/ml) on the glycogen content in bovine mesenteric arteries after incubation for 30 to 180 min in a medium containing 5 6 mM glucose A *significant* ($P < 0\,02$) *increase in glycogen content by insulin was found after* 180 min Mean±S E M (n=11)

Effect of insulin on glycogen content The effect of insulin on the glycogen content in bovine mesenteric arteries and rabbit colon smooth muscle was determined after an incubation period of 180 min in 5 6 mM glucose In both tissues insulin was found to increase the glycogen content In bovine mesenteric arteries the glycogen content rose by 26 9±5 4 mg/100 g (n=11, $P < 0\,001$) from a control value of 118 5±16 2 mg/100 g and in rabbit colon

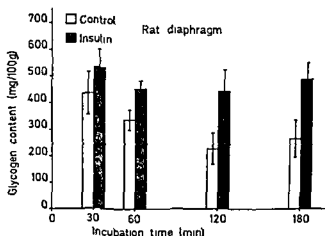


Fig 1b Effect of insulin (0 1 U/ml) on the glycogen content in rat hemidiaphragm after incubation times of 30 to 180 min The concentration of glucose in the medium was 5 6 mM The effect of insulin was significant ($P < 0\,05$ or less) at all incubation times. Mean±S E M (n=6)

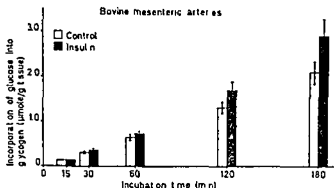


Fig 2a. Effect of insulin (0.1 U/ml) on the incorporation of glucose-^{14}C into glycogen in bovine mesenteric arteries determined after incubation periods of 15 to 180 min. The concentration of glucose in the medium was 5.6 mM. The asterisks denote the significance of the effect *=P < 0.05, **=P < 0.01, ***=P < 0.001. Mean±S.E.M. (n=10)

smooth muscle it rose by 20.3±4.4 mg/100 g (n=10, P < 0.01) from a control value of 47.0±5.2 mg/100 g.

The time course for the effect of insulin on the glycogen content was studied in bovine mesenteric arteries and for comparison also in the intact rat hemidiaphragm preparation. The effect of insulin on the arterial tissue became significant after 180 min (fig 1a) while in rat diaphragm, where the action of insulin was more pronounced, the effect was significant after 30 min (fig 1b).

Effect of insulin on the incorporation of glucose-^{14}C into glycogen. Insulin stimulated the incorporation of glucose-^{14}C into glycogen in bovine mesen-

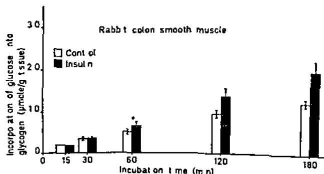


Fig 2b. Incorporation of glucose-^{14}C into glycogen in rabbit colon smooth muscle; experimental conditions were the same as in fig 2a. Statistical significance is as in fig 2a. Mean±S.E.M. (n=10)

Table 2

Effect of variations in the extracellular Na^+ and K^+ concentrations on the incorporation of glucose ^{14}C into glycogen in bovine mesenteric arteries The tissue samples were incubated for 180 min in 5 6 mM glucose ^{14}C Except for Na^+, K^+ the ionic composition of the medium was that of Krebs Henseleit bicarbonate buffer Mean ± S E M (n=8)

Na (mM)	K (mM)	Incorporation of glucose ^{14}C into glycogen (µmol/g tissue)		
		Control	Insulin	Effect
150	0	1 63 ± 0 32	2 45 ± 0 34	0 82 ± 0 14 $P < 0 001$
145	5	1 61 ± 0 23	2.52 ± 0 39	0 91 ± 0.21 $P < 0 01$
135	15	1 60 ± 0 30	2.37 ± 0 41	0 77 ± 0 18 $P < 0 02$
100	50	1 63 ± 0 31	2 03 ± 0 37	0 40 ± 0 12 $P < 0 02$

teric arteries and rabbit colon smooth muscle (fig 2a and b) A significant effect was noted after an incubation period of 30 min in the arterial tissue and after 60 min in the rabbit colon smooth muscle The effect increased with time and after 180 min it was 38 5 ± 9 5 % in bovine mesenteric arteries and 60 0 ± 18 8 % in rabbit colon smooth muscle as compared to the control values

Effect of extracellular Na^+ and K^+ on the incorporation of glucose-^{14}C into glycogen When tissue metabolism is studied under *in vitro* conditions the Na^+ and K^+ content of the medium may influence the glycogen metabolism (CLAUSEN 1972) From table 2 it is seen that in bovine mesenteric arteries, the variations in the K^+ of the medium within the range 0–50 mM with a corresponding change in the Na^+ had no effect on the incorporation of glucose ^{14}C into glycogen in the control preparations Insulin stimulated the incorporation of glucose at all Na^+, K^+ concentrations At a K^+ concentration of 50 mM the effect of insulin was reduced but the decrease was not significant

Effect of insulin on glycogen synthetase and phosphorylase From table 3 it is seen that insulin tended to increase the glycogen synthetase I activity in bovine mesenteric arteries ($P < 0 05$) and in rabbit colon smooth muscle ($P < 0 1$) The total glycogen synthetase activity was not affected by insulin in either tissue The ratio of glycogen synthetase I to total glycogen synthetase activity was increased by insulin in rabbit colon smooth muscle ($P < 0 02$) while no significant change was found in the mesenteric arteries The values for glycogen synthetase I and total glycogen synthetase activity were similar in the two types of tissue

Table 3

Influence of insulin (0.1 U/ml) on glycogen synthetase activity in bovine mesenteric arteries and rabbit colon smooth muscle. The enzyme activity was assayed after incubation for 180 min. in a medium containing 5.6 mM glucose. Glycogen synthetase activity is expressed as µmol UDPG/g wet tissue per hour. Mean ± S.E.M. Numbers of observations are given in brackets

Preparation	Experimental conditions	Glycogen synthetase activity		
		No Glc-6-P	Glc-6-P added	% I form
Bovine mesenteric arteries (n=11)	control	0.48±0.06	5.66±0.93	9.7±1.3
	insulin	0.70±0.11	6.47±0.95	10.8±0.8
	effect	0.23±0.09	0.81±0.85	1.2±1.1
		$P<0.05$	N.S.	N.S.
Rabbit colon smooth muscle (n=12)	control	0.52±0.08	4.62±0.52	11.3±1.20
	insulin	0.65±0.07	4.75±0.48	14.1±1.59
	effect	0.12±0.07	0.13±0.24	2.8±1.0
		$P<0.1$	N.S.	$P<0.02$

The phosphorylase *a* and total phosphorylase activities were not influenced by insulin in either of the examined tissues when the enzyme activity was determined after incubation of the tissues in 5.6 mM glucose for 180 min. Expressed as µmol glucose-1-phosphate/g wet tissue per hr, the phosphorylase *a* activity was 2.0±0.3 (n=11) in bovine mesenteric arteries, 8.7±1.1 (n=12) in rabbit colon smooth muscle, and the total phosphorylase activity was 8.2±0.9 and 196.3±26.1, respectively. The total activity was thus about 25 times higher in rabbit colon smooth muscle than in bovine mesenteric arteries and the phosphorylase *a* activity was 4–5 times higher in the former.

Comparison between the effect of insulin and glucose concentration on glucose uptake and glycogen content in bovine mesenteric arteries. The effect of insulin and of glucose concentration on the glucose uptake and glycogen content was determined on the same mesenteric arteries. From fig. 3 it is seen that insulin increased the glycogen content at a medium concentration of 2.8 mM glucose while having no significant effect on the glucose uptake. Raising the medium concentration of glucose to 11.1 mM doubled the glucose uptake but the glycogen content was not proportionally affected. These results indicate that the effect of insulin on the glycogen content in bovine mesenteric arteries cannot be explained by its effect on glucose uptake only.

The effect of insulin on the glycogen breakdown was also studied. P

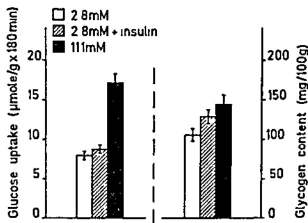


Fig 3 Comparison between the effect of glucose concentration and insulin (0 1 U/ml) on the glucose uptake and glycogen content in bovine mesenteric arteries The glucose uptake and glycogen content were determined on the same samples after an incubation period of 180 min Mean ± S E M (n=7)

mesenteric arteries were incubated for 180 min with and without insulin in a medium containing no glucose The initial glycogen content was 117 7±13 0 mg/100 g (n=9) After the incubation period the glycogen content was 73 6±8 4 mg/100 g without insulin and 76 2±9 4 mg/100 g with added insulin The glycogenolysis was therefore unaffected by insulin and this result is in agreement with the absence of an insulin effect on phosphorylase activity

Effect of insulin on lactate metabolism To investigate if the increase by insulin of the glucose uptake of bovine mesenteric arteries and rabbit colon smooth muscle stimulated glycolysis, the lactate elimination was determinated after incubation for 180 min in 5 6 mM glucose In both tissues the lactate elimination was increased by insulin In bovine mesenteric arteries the lactate elimination, expressed as µmol/g tissue×180 min, increased by 1 8±0 3 (n=7, P < 0 01) from a control value of 8 4±0 7 and in rabbit colon smooth muscle, it increased by 9 7±2 9 (n=12, P < 0 01) from a control value of 40 5 ± 3 4 The lactate content of the tissues was not influenced by insulin

Discussion

The glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle was increased by insulin (0 1 U/ml) after an incubation period of

180 min and at a glucose concentration of 5 6 mM in the medium The membrane transport of glucose was previously found to be a rate limiting step in the glucose utilization in these tissues and in both of them the membrane transport of monosaccharide was stimulated by insulin (Arnqvist 1971, 1972) It therefore seems probable that the increase in the glucose uptake produced by insulin is caused by a stimulation of glucose transport The increase in the glucose uptake by insulin was 26 2 ± 8 7 % in bovine mesenteric arteries and 14 0 ± 3 5 % in rabbit colon smooth muscle After an incubation period of 60 min the glucose uptake in rat soleus muscle was increased about 100 % by insulin (0 1 U/ml) (Chaudry & Gould 1969), while in bovine mesenteric arteries and rabbit colon smooth muscle no effect was found after this time

Lundholm & Mohme Lundholm (1963) found that insulin increased the glycogen content in bovine mesenteric arteries and rabbit stomach muscle The present results on bovine mesenteric arteries and rabbit colon smooth muscle confirm their observation In comparison with rat diaphragm the effect of insulin on the glycogen content in bovine mesenteric arteries was weaker and appeared later

Insulin increased the incorporation of glucose-^{14}C into glycogen in bovine mesenteric arteries and rabbit colon smooth muscle This effect appeared after 30 min in bovine mesenteric arteries and after 60 min. in rabbit colon smooth muscle At the end of the incubation period, when the action of insulin was most pronounced, the increase was 38 5 ± 9 5 % in the former tissue and 60 0 ± 18 8 % in the latter tissue After 15 min the incorporation of glucose into glycogen was increased about threefold by insulin in rat diaphragm (Carlin & Hechter 1965) and sevenfold in rat levator ani muscle (Adolfsson 1973)

Raising the K^+ of the medium from 0 to 50 mM, with a corresponding reduction of the Na^+, had no effect on the incorporation of glucose into glycogen in bovine mesenteric arteries, while the effect of insulin tended to decrease These results on bovine mesenteric arteries are similar to those of Stadie & Zapp (1947) on the influence of K^+ and Na^+ on glycogen synthesis in the rat diaphragm

In skeletal muscle the effect of insulin on glycogen synthesis is greater than could be expected from the stimulation of glucose transport only (Larner *et al* 1959, Norman *et al* 1959) This is due to an increase in the glycogen synthetase I activity (Villar Palasi & Larner 1961) The ratio of glycogen synthetase I to total glycogen synthetase activity was increased by insulin in rabbit colon smooth muscle Insulin increased the glycogen synthetase I activity in bovine mesenteric arteries and tended to have the same action in rabbit colon smooth muscle When the effects of insulin of glucose concentration or [illegible]

compared in bovine mesenteric arteries, insulin was found to increase the glycogen content at a glucose concentration of 2 8 mM, while having no significant effect on the glucose uptake The increase in the glycogen content without an increase in the glucose uptake indicates a direct action of insulin on the glycogen metabolism Since the phosphorylase activity or glycogenolysis was not influenced by insulin, the increase in glycogen content was probably due to the increased glycogen synthetase I activity

As in rat skeletal muscle (Clausen 1968) the effect of insulin on the glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle was accompanied by an increase in glycolysis

In conclusion, the results of the present study show that the effects of insulin on glucose metabolism in bovine mesenteric arteries and rabbit colon smooth muscle are qualitatively similar to those in rat skeletal muscle, *although they are less pronounced and appear later*

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Binding of 2,4-Dichloro- and 2, 4, 5-Trichlorophenoxyacetic Acid to Bovine Serum Albumin

By

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Abstract The binding of 2 4 dichloro and 2,4,5 trichlorophenoxyacetic acid (2,4-D and 2,4,5 T) to bovine serum albumin has been studied by a gel filtration technique (HUMMEL & DREYER 1952) Both compounds were extensively bound to albumin 2,4,5 T to a higher extent than 2,4-D The binding of both herbicides was reduced when the protein contained palmitic acid.

Key words Herbicides – binding to serum albumin

Previous studies have shown that 2,4-dichlorophenoxyacetic acid (2,4 D) inhibits the growth of L 929 cells in monolayer cultures and induces a transient accumulation of cytoplasmic triglyceride particles (KOLBERG *et al* 1971, KOLBERG *et al* 1972) The fatty acids of the accumulated triglycerides were largely of exogenous origin An increased uptake of fatty acids in cultured cells has also been found on exposure to the structurally related *p* chlorophenoxyisobutyrate (SPECTOR & SOBOROFF 1971), used in the treatment of hyperlipaemia It was suggested that this was due to a displacement of fatty acids from strong to weaker binding sites on human plasma albumin, thereby making fatty acids more available to the cells

The gel filtration experiments by ERNE (1966) indicated that 2,4-D was bound to plasma proteins A competition between 2,4-D and fatty acids for binding sites on serum albumin could thus influence the utilization of fatty acids in cell cultures and, at least partly, explain the accumulation of triglycerides The binding of 2,4 D to bovine serum albumin (BSA) and the relationship between this interaction and fatty acids has therefore been examined by the gel filtration method described by HUMMEL & DREYER (1962), for the determination of reversible complex formation A comparison between the binding of 2,4 D and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) to BSA has also been made

Materials and Methods

2,4-dichlorophenoxy 2-^{14}C-acetic acid (41 mci/mmol), 2,4,5 trichlorophenoxy 1 ^{14}C acetic acid (30 mci/mol), 9,10-^{3}H palmitic acid (500 mci/mmol), 1,2-^{3}H n hexadecane and 1 ^{14}C-n hexadecane were supplied by The Radiochemical Centre, Amersham

Reference preparations of 2,4-D and 2,4,5 T (purities > 99 4 and > 99 % respectively, gas chromatography by the manufacturer) were gifts from Koge Chemical Works, Ltd, Koge, Denmark Crystallized and lyophilized bovine serum albumin was purchased from Sigma Chemical Company (Lot No 80C 8030) The molecular weight was assumed to be 69,000 Palmitic acid (purity > 99 %) was obtained from the Hormel Institute, Austin, Minn

Na K salt of the herbicides

A stock solution containing 25 mg 2,4-D/ml was prepared as described previously (KOLBERG *et al* 1971) A stock solution of the less soluble 2,4,5 T contained 7 2 mg/ml Care was taken to ensure that the ionic composition was the same at different dilutions of the herbicides

The commercial benzene solution containing ^{14}C-2,4-D or ^{14}C-2,4 5 T was evaporated to dryness under a stream of nitrogen at 50° The residues were dissolved in aqueous NaOH KOH (0 01 N 0 01 N) and added to the elution buffer The activity in the eluant was in the range of 1×10^{-2} to 5×10^{-2} µci/ml

Addition of palmitic acid to defatted BSA

Celite® was coated with palmitic acid of specific activity 100 µci/mmol and incubated with a solution of BSA (SPECTOR & HOAK 1969) The albumin had been treated with charcoal to remove inherent fatty acids (CHEN 1967) The amount of fatty acid taken up by the BSA solution was determined by radioactivity measurements

The concentration of protein was measured by the method of LOWRY *et al* (1951)

Gel filtrations

A 0 9 × 18 cm column of Sephadex G 50, fine, was equilibrated with 75 mM Tris HCl, pH 7 4, containing ^{14}C-labelled 2,4-D or 2,4,5 T at the same concentration and specific radioactivity as in the subsequent sample The protein herbicide samples (300 µl) were equilibrated at room temperature for at least 30 min before application to the column Elution was carried out with the equilibration buffer at a flow rate of about 0 3 ml/min Fractions of 0 6 ml were collected

Radioactivity measurements

50 µl from each column fraction was treated with 0 5 ml Hyamine 10-X® for 2 hours at 37° in a rotating polyethylene vial, then counted in 15 ml scintillation liquid (4 g PPO, 0 005 g dmPOPOP, 1000 ml toluene and 2 ml acetic acid)

Counting was performed in a Packard Tri Carb spectrometer, model 3365, operated at 3°, using ^{3}H and ^{14}C-n hexadecane as reference materials

Calculations

Bound herbicide determined from the peak area was related to the amount of protein applied to the column The binding data were plotted as $\bar{v}$ against $\bar{v}/c$ (SCATCHARD 1949) where v is the average number of moles bound herbicide and c is the concentration of free herbicide. The binding sites and the association constants were obtained from the intercepts according to the graphic parameter fitting method of BERSON & YALOW (1959)

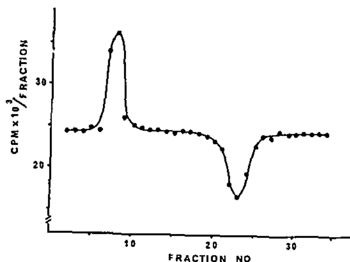


Fig 1 Elution pattern for measurement of the binding of 2 4-5 T to BSA on a column of Sephadex G 50 fine The concentration of 2 4 5 T in both sample and eluant was 1 1 mM and the amount protein used was 6 9 mg

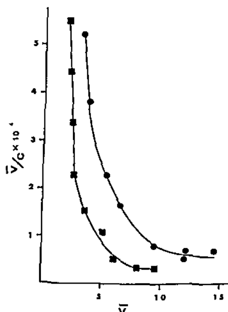


Fig 2 Scatchard plots for the binding of 2 4-D and 2 4 5 T by BSA $\bar{v}$ represents the number of mol herbicide bound per mol of protein and c the concentration of free herbicide The amount of protein used was 2 6 3 5 or 6 9 mg The concentration of 2 4-D varied between 34 µM and 2 7 mM The concentration of 2,4,5 T was in the range of 59 µM to 2 3 mM

■ – ■ 2 4 D ● – ● 2 4 5 T

Table 1.

Binding of 2,4-D and 2,4,5-T to native BSA*

Ligand	n_1	$K_1\ M^{-1}$	n_2	$K_2\ M^{-1}$
2,4-D	3	4×10^4	40	1×10^2
2,4,5-T	5	3×10^4	70	1×10^2

* The association constants obtained are apparent constants not corrected for electrostatic interactions and other factors

Results

The elution pattern (fig 1) shows the increased concentration of 2,4,5-T associated with BSA and the subsequent fall below the base line representing the part of the herbicide abstracted by the albumin The through area should have equalled the peak area but was found to be about 90 % of the latter for both herbicides

As shown in fig 2, the SCATCHARD plots demonstrated that BSA contained at least two classes of binding sites for both 2,4-D and 2,4,5-T The sharply ascending limb at the lower $\bar{v}$ values indicates a high-affinity class, the nearly horizontal limb at the higher $\bar{v}$ values a low-affinity class The data were fitted to a model with two classes of binding sites (table 1) The binding parameters show that the high affinity group (n_1) contains a small number of binding sites, whereas the low-affinity group (n_2) contains a large number of binding sites for the herbicides The data show that 2,4,5-T is bound to BSA to a higher extent than 2,4-D

The amount of bound herbicide was reduced by 40 and 29 % for 2,4-D and 2,4,5-T respectively when 7 mol of palmitic acid was added per mol of defatted BSA (table 2)

Table 2

Effect of palmitic acid on the binding of 2,4-D or 2,4,5 T to bovine serum albumin*

Ligand (2 3 mM)	Mol bound ligand per mol BSA			
	Defatted BSA		Fatty acid BSA 7 1	
2,4 D	7 1	8 3	4 4	4 8
2 4 5 T	13	14	9 2	11

* The amount of BSA in each sample (300 µl) was 6 9 mg

Discussion

The herbicides are extensively bound by native BSA The interactions are probably reversible as the gel filtration experiments by ERNE (1966), who used buffer without 2,4-D as eluant, suggests only a weak binding of 2,4-D by plasma proteins

2,4,5-T is bound to BSA to a higher extent than 2,4-D The rate of disappearance of 2,4,5-T (free acid) from the body of mice is much slower than for 2,4-D (free acid and esters) (ZIELINSKI & FISHBEIN 1967) This may partly depend on the observed difference in protein binding for these herbicides

The amounts of 2,4-D and 2,4,5-T bound to albumin are reduced when 7 mol of palmitic acid is added per mol BSA This is similar to the highest molar ratios observed in animals or in man (DI GIROLAMO *et al* 1961, GALLIN *et al* 1969) This reduced binding could be due to competition between the herbicides and fatty acids for the same sites on albumin and/or that the binding of fatty acids may have induced structural changes in the albumin molecule which alter the affinity for 2,4-D and 2,4,5-T These results thus suggest that the free fatty acid concentration in blood may affect the capacity of albumin for transport of the phenoxy herbicides in the organism Inhibitory effects of free fatty acids (molar ratios 3 5 and 7) on the binding properties of BSA for eight different drugs have been published by RUDMAN *et al* (1971)

The interaction between the herbicides and albumin may also alter the binding properties of albumin for fatty acids and, hence, influence the utilization of fatty acids in cell cultures

In vivo, a binding of 2,4-D and 2,4,5-T to albumin could act as a mechanism for preventing acute toxicity These interactions could, however, tend to prolong the toxic effect These problems, as well as the observed interference by fatty acids and its possible impact on the treatment of herbicide poisoning can only be solved by animal experiments

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Studies on the Effects of some Local Anaesthetics on the Uptake of ^{3}H-l-Noradrenaline into Vascular and Cardiac Tissues *in Vitro*

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Key words Blood vessel – heart – local anaesthetics – uptake of noradrenaline

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The local anaesthetic compound cocaine contracts vascular smooth muscle probably by inhibiting the uptake of noradrenaline into adrenergic nerves thereby making more noradrenaline available at the effector sites (MUSCHOLL 1961, FURCHGOTT *et al* 1963, KOPIN 1964) This inhibition has been shown to occur at the level of the cell membrane of the adrenergic neuron (HILLARP & MALMFORS 1964, CARLSSON & WALDECK 1965) Two other local anaesthetics – mepivacaine and lidocaine – have also been shown to contract isolated blood vessels (ABERG & WAHLSTROM 1972, ABERG 1972) A possible mechanism for the contracting action of one of these local anaesthetic compounds, mepivacaine has been suggested by JORFELDT *et al* (1970) to be an inhibitory effect of the compound on the neural uptake of noradrenaline In other experiments however, it has been shown that the contracting effect of mepivacaine is not inhibited by adrenergic α-blockers *in vitro* (ÅBERG & WAHLSTROM 1972) or *in vivo* (ABERG & DHUNER 1972) The contracting effect on isolated rat portal veins of both cocaine and lidocaine was recently found not to be inhibited by the adrenergic α receptor blocking drug dibenzyline (unpublished results) This was in agreement with the finding of DANIEL & WOLOWYK (1966) who showed that the cocaine induced contractures of isolated rabbit uteri were not inhibited by dibenzyline

In the present report we describe some experiments on the effects of cocaine, mepivacaine and lidocaine on the uptake of ^{3}H l noradrenaline into isolated guinea pig aorta and cardiac tissue The optically active isomers of mepivacaine have been included in the experiments, as L(+) mepivacaine

is more potent as a vasoconstrictor than D(−) mepivacaine (ABERG & WAHLSTROM 1972) Lu 3-010 (3,3-dimetyl-1-(3 metylaminopropyl) phenylphtalan) has been used as this compound is known to be an efficient and specific inhibitor of the uptake of noradrenaline (WALDECK 1968)

Material and Methods

^{3}H l noradrenaline (^{3}H NA) with a specific activity of 6 6 ci/mmol was obtained from The Radiochemical Center, Amersham, England The radiochemical purity of the substance was confirmed by radiopaper chromatography in isopropanol 2 N hydrochlorid acid (65 35)

Guinea pigs weighing 300–500 g were rapidly killed by stunning and bleeding and their hearts and aortas were placed in a modified Krebs solution containing (g/l) NaCl 7 13 KCl 0 35, $CaCl_2$ 0 28, $MgCl_2 \times 5H_2O$ 0 24, $NaHCO_3$ 1 30, KH_2PO_4 0 16 and glucose 2.07 Ascorbic acid (50 µg/ml) was added to the solution which was aerated with a mixture of 93 5 % O_2 + 6 5 % CO_2 In order to inhibit the active uptake of noradrenaline some heart and aortic preparations were tested at 0° in parallel with the other experiments which were all performed at 37°

The heart preparation consisted of the isolated auricles and connective tissues while the isolated aortas used in this investigation consisted of strips of the thoracic aorta The preparations were allowed to stabilize for 30 min before addition of the compounds investigated After another 30 min of incubation of the tissue and in the presence of the test compound 40 µl of a solution containing 35 µg l noradrenaline and 2 5 µci ^{3}H NA in 2 ml of 0 01 M HCl was added Thus the concentration of l noradrenaline in the bath was 1×10^{-7} M After 30 min exposure to the tritiated solution the preparations were washed in Krebs solution, dried weighed and dissolved in 2 ml soluene Eighteen ml of a scintillation fluid containing 3 g diphenyl oxazole (PPO) and 0 3 g 2,2 p phenylene bis (5 phenyloxazole) (POPOP) per 1 l of toluene was added and the radioactivity was measured in a Packard Tri Carb scintillation counter The counting efficiency was 14–18 per cent for the heart preparations and 21–25 per cent for the isolated aortas

Results and Discussion

In both the heart auricles (fig 1) and the aorta (fig 2) there was an active uptake and retention of radioactivity, representing a larger fraction of the total in the former (82 %) than in the latter (48 %) tissue This active uptake appeared to be almost completely blocked in the heart and also largely in the aorta by Lu 3-010 and by cocaine None of the other compounds tested had a significant effect on the uptake and retention of radioactivity in the heart and aorta on incubation with ^{3}H NA

At low concentrations of ^{3}H-NA (10^{-7} M) in the incubation total radioactivity taken up and retained by adrenergically

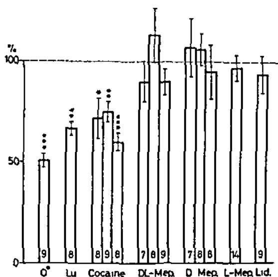


Fig 1 Uptake of ³H l noradrenaline by isolated guinea pig auricles, expressed as per cent of control values at 37° 0° denotes results from control experiments performed at 0° Lu denotes Lu 3 010 Cocaine, DL mepivacaine and D mepivacaine were tested in the following concentrations (from left to right in the figure) 1×10^{-6} M 1×10^{-5} M and 1×10^{-4} M Lu 3-010, L mepivacaine and lidocaine were tested in the concentration 1×10^{-4} M Each bar shows the mean ± S E M from the number of experiments shown within the bar The significance of the differences between the control groups and the test groups are shown in the figure, where * means $P < 0.05$, ** means $P < 0.01$ and *** means $P < 0.001$

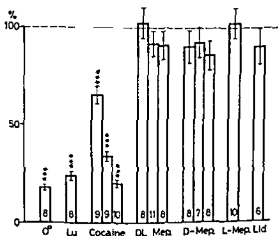


Fig 2. Uptake of ³H l noradrenaline by isolated guinea pig aortas in per cent of control values at 37° All symbols are the same as in fig 1 The concentrations of the inhibitors tested were (from left to right in the figure) Lu 3 010 1×10^{-4} M, cocaine, DL-mepivacaine and D mepivacaine 1×10^{-5} M, 1×10^{-4} M and 1×10^{-3} M, L mepivacaine and lidocaine 1×10^{-3} M

consists mainly of unchanged ^{3}H-NA located in the adrenergic nerves (ALMGREN & JONASON 1971, FARNEBO 1971) With a lower density of adrenergic innervation the contribution of metabolites to the total radioactivity increases and thus the specifity of the uptake decreases (ALMGREN & JONASON 1971) This condition may explain why the sensitivity to Lu 3 010 and cocaine was higher in the heart with its denser innervation than in the aorta

However, the present data clearly indicate that neither lidocaine nor the enantiomorphs of mepivacaine had any significant effects on the amine-uptake mechanism of the adrenergic neuron This finding and the failure of α blocking agents to inhibit the contracting effects of lidocaine and mepivacaine supports the view that contractions brought about by these local anaesthetics are mediated via non adrenergic mechanisms (ÅBERG & WAHLSTROM 1972) As an α blocking agent did not inhibit cocaine induced contractures it may be questioned whether the contracting effect of cocaine is related to an inhibition of the uptake of noradrenaline into adrenergic nerves

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